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(54) Title: ATTENUATED HUMAN-BOVINE CHIMERIC PARAINFLUENZA VIRUS (PIV) VACCINES

(57) Abstract: Chimeric human-bovine parainfluenza viruses (PIVs) are infectious and attenuated in humans and other mammals and useful individually or in combination in vaccine formulations for eliciting an anti-PIV immune response. Also provided are isolated polynucleotide molecules and vectors incorporating a chimeric PIV genome or antigenome which includes a partial or complete human or bovine PIV "background" genome or antigenome combined or integrated with one or more heterologous gene(s) or genome segment(s) of a different PIV. Chimeric human-bovine PIV of the invention include a partial or complete "background" PIV genome or antigenome derived from or patterned after a human or bovine PIV virus combined with one or more heterologous gene(s) or genome segment(s) of a different PIV virus to form the human-bovine chimeric PIV genome or antigenome. In certain aspects of the invention, chimeric PIV incorporate a partial or complete human PIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a bovine PIV, whereby the resultant chimeric virus is attenuated by virtue of host-range restriction. In alternate embodiments, human-bovine chimeric PIV incorporate a partial or complete bovine PIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV gene that encode a human PIV immunogenic protein, protein domain or epitope, for example encoded by PIV HN and/or F glycoprotein gene(s) or genome segment(s). Human-bovine chimeric PIV of the invention are also useful as vectors for developing vaccines against other pathogens. A variety of additional mutations and nucleotide modifications are provided within the human-bovine chimeric PIV of the invention to yield desired phenotypic and structural effects.

# ATTENUATED HUMAN-BOVINE CHIMERIC PARAINFLUENZA VIRUS (PIV) VACCINES

## CROSS-REFERENCES TO RELATED APPLICATIONS

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This application claims the benefit of United States Provisional Patent Application Serial No. 60/143,134, filed by Bailly et al. on July 9, 1999.

## BACKGROUND OF THE INVENTION

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Human parainfluenza virus type 3 (HPIV3) is a common cause of serious lower respiratory tract infection in infants and children less than one year of age. It is second only to respiratory syncytial virus (RSV) as a leading cause of hospitalization for viral lower respiratory tract disease in this age group (Collins et al., in B. N. Fields

15    Virology, p. 1205-1243, 3rd ed., vol. 1., Knipe et al., eds., Lippincott-Raven Publishers, Philadelphia, 1996; Crowe et al., Vaccine 13:415-421, 1995; Marx et al., J. Infect. Dis. 176:1423-1427, 1997, all incorporated herein by reference). Infections by this virus result in substantial morbidity in children less than 3 years of age. HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup) and also can cause

20    severe pneumonia and bronchiolitis (Collins et al., 1996, supra). In a long term study over a 20-year period, HPIV1, HPIV2, and HPIV3 were identified as etiologic agents for 6.0, 3.2, and 11.5%, respectively, of hospitalizations for respiratory tract disease accounting in total for 18% of the hospitalizations, and, for this reason, there is a need for an effective vaccine (Murphy et al., Virus Res. 11:1-15, 1988). The parainfluenza viruses

25    have also been identified in a significant proportion of cases of virally-induced middle ear effusions in children with otitis media (Heikkinen et al., N. Engl. J. Med. 340:260-264, 1999, incorporated herein by reference). Thus, there is a need to produce a vaccine against these viruses that can prevent the serious lower respiratory tract disease and the otitis media that accompanies these HPIV infections. HPIV1, HPIV2, and HPIV3 are

30    distinct serotypes that do not elicit significant cross-protective immunity. The major protective antigens of PIVs are the hemeagglutinin (HN) and fusion (F) glycoproteins, which mediate viral attachment, penetration and release. Protection against reinfection is mediated primarily by virus-neutralizing antibodies.

Despite considerable efforts to develop effective vaccine therapies against HPIV, approved vaccine agents that prevent HPIV related illness have not yet been achieved. The most promising prospects to date are live attenuated vaccine viruses since these have been shown to be efficacious in non-human primates even in the presence of

5  passively transferred antibodies, an experimental situation that simulates that present in the very young infant who possesses maternally acquired antibodies (Crowe et al., 1995, *supra*; and Durbin et al., J. Infect. Dis. 179:1345-1351, 1999a; each incorporated herein by reference). Two live attenuated PIV3 vaccine candidates, a temperature-sensitive (*ts*) derivative of the wild type PIV3 JS strain (designated PIV3*cp*45) and a bovine PIV3

10  (BPIV3) strain, are undergoing clinical evaluation (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; Karron et al., 1995a, *supra*; Karron et al., 1995b, *supra*; each incorporated herein by reference). The BPIV3 vaccine candidate is attenuated, genetically stable and immunogenic in human infants and children. A second PIV3 vaccine candidate, JS cp45, is a cold-adapted mutant of the JS wildtype (wt) strain of

15  HPIV3 (Karron et al., 1995b, *supra*; and Belshe et al., J. Med. Virol. 10:235-242, 1982a; each incorporated herein by reference). This live, attenuated, cold-passaged (cp) PIV3 vaccine candidate exhibits temperature-sensitive (ts), cold-adaptation (ca), and attenuation (att) phenotypes, which are stable after viral replication *in vivo*. The cp45 virus is protective against human PIV3 challenge in experimental animals and is

20  attenuated, genetically stable, and immunogenic in seronegative human infants and children (Belshe et al., 1982a, *supra*; Belshe et al., Infect. Immun. 37:160-165, 1982b; Clements et al., J. Clin. Microbiol. 29:1175-1182, 1991; Crookshanks et al., J. Med. Virol. 13:243-249, 1984; Hall et al., Virus Res. 22:173-184, 1992; Karron et al., 1995b, *supra*; each incorporated herein by reference). Because these PIV3 candidate vaccine

25  viruses are biologically derived there is no proven method for adjusting their level of attenuation as will likely be necessary for broad clinical application.

To facilitate development of PIV vaccine candidates, recombinant DNA technology has recently made it possible to recover infectious negative-strand RNA viruses from cDNA (for reviews, see Conzelmann, J. Gen. Virol. 77:381-389, 1996;

30  Palese et al., Proc. Natl. Acad. Sci. U.S.A. 93:11354-11358, 1996; each incorporated herein by reference). In this context, rescue of recombinant viruses has been reported for infectious respiratory syncytial virus (RSV), rabies virus (RaV), simian virus 5 (SV5), rinderpest virus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), measles virus (MeV), mumps virus (MuV) and Sendai virus (SeV) from cDNA-encoded

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antigenomic RNA in the presence of essential viral proteins (see, e.g., Garcin et al., EMBO J. 14:6087-6094, 1995; Lawson et al., Proc. Natl. Acad. Sci. U.S.A. 92:4477-4481, 1995; Radecke et al., EMBO J. 14:5773-5784, 1995; Schnell et al., EMBO J. 13:4195-4203, 1994; Whelan et al., Proc. Natl. Acad. Sci. U.S.A. 92:8388-8392, 1995;

5   Hoffman et al., J. Virol. 71:4272-4277, 1997; Kato et al., Genes to Cells 1:569-579, 1996, Roberts et al., Virology 247:1-6, 1998; Baron et al., J. Virol. 71:1265-1271, 1997; International Publication No. WO 97/06270; Collins et al., Proc. Natl. Acad. Sci. USA 92:11563-11567, 1995; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to published International Application No. WO 98/02530 and priority

10   U.S. Provisional Application Nos. 60/047,634, filed May 23, 1997, 60/046,141, filed May 9, 1997, and 60/021,773, filed July 15, 1996); U.S. Patent Application Serial No. 09/291,894, filed on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999; U.S. Provisional Patent Application Serial No. 60/143,097, filed by Bucholz et al. on July 9, 1999; Juhasz et al., J. Virol. 71:5814-5819,

15   1997; He et al. Virology 237:249-260, 1997; Peters et al. J. Virol. 73:5001-5009, 1999; Whitehead et al., Virology 247:232-239, 1998a; Whitehead et al., J. Virol. 72:4467-4471, 1998b; Jin et al. Virology 251:206-214, 1998; Bucholz et al. J. Virol. 73:251-259, 1999; Whitehead et al., J. Virol. 73:3438-3442, 1999, and Clarke et al., J. Virol. 74:4831-4838, 2000; each incorporated herein by reference in its entirety for all purposes).

20         In more specific regard to the instant invention, a method for producing HPIV with a wt phenotype from cDNA was recently developed for recovery of infectious, recombinant HPIV3 JS strain (see, e.g., Durbin et al., Virology 235:323-332, 1997a; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application Serial No. 60/047,575, filed May 23, 1997 (corresponding to International

25   Publication No. WO 98/53078), and U.S. Provisional Application Serial No. 60/059,385, filed September 19, 1997, each incorporated herein by reference). In addition, these disclosures allow for genetic manipulation of viral cDNA clones to determine the genetic basis of phenotypic changes in biological mutants, e.g., which mutations in the HPIV3 cp45 virus specify its ts, ca and att phenotypes, and which gene(s) or genome segment(s)

30   of BPIV3 specify its attenuation phenotype. Additionally, these and related disclosures render it feasible to construct novel PIV vaccine candidates having a wide range of different mutations and to evaluate their level of attenuation, immunogenicity and phenotypic stability (see also, U.S. Provisional Patent Application Serial No. 60/143,134,

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filed by Bailly et al. on July 9, 1999; and U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999; each incorporated herein by reference).

Thus, infectious wild type recombinant PIV3 (r)PIV3, as well as a number of *ts* derivatives, have now been recovered from cDNA, and reverse genetics systems

5   have been used to generate infectious virus bearing defined attenuating mutations and to study the genetic basis of attenuation of existing vaccine viruses. For example, the three amino acid substitutions found in the L gene of *cp45*, singularly or in combination, have been found to specify the *ts* and attenuation phenotypes. Additional *ts* and attenuating mutations are present in other regions of the PIV3 *cp45*. In addition a chimeric PIV1

10  vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45*L (Skiadopoulos et al., J. Virol. 72:1762-1768, 1998; Tao et al., J. Virol. 72:2955-2961, 1998; Tao et al., Vaccine

15  17:1100-1108, 1999, incorporated herein by reference). rPIV3-1.cp45L was attenuated in hamsters and induced a high level of resistance to challenge with PIV1. A recombinant chimeric virus, designated rPIV3-1.*cp45*, has been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations that occur in HN and F, and is highly attenuated in the upper and lower respiratory tract of hamsters (Skiadopoulos et al.,

20  Vaccine 18:503-510, 1999a).

BPIV3, which is antigenically-related to HPIV3, offers an alternative approach to the development of a live attenuated virus vaccine for HPIV1, HPIV2, and HPIV3. The first vaccine used in humans, live vaccinia virus believed to be of bovine origin, was developed by Jenner almost 200 years ago for the control of smallpox.

25  During the ensuing two centuries, vaccinia virus was successful in controlling this disease and played an essential role in the final eradication of smallpox. In this "Jennerian" approach to vaccine development, an antigenically-related animal virus is used as a vaccine for humans. Animal viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. At present, there is a lack of

30  a thorough understanding regarding the genetic basis for this form of host range restriction. Evolution of a virus in its mammalian or avian host results in significant divergence of nucleotide (nt) and amino acid sequences from that of the corresponding sequences in the related human virus. This divergent sequence, consisting of a large number of sequence differences, specifies the host range attenuation phenotype. Having

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an attenuation phenotype which is based on numerous sequence differences is a desirable property in a vaccine virus since it should contribute to the stability of the attenuation phenotype of the animal virus following its replication in humans.

The recently licensed quadrivalent rotavirus is an example of the Jennerian

5   approach to vaccine development in which a nonhuman rotavirus strain, the rhesus rotavirus (RRV), was found to be attenuated in humans and protective against human serotype 3 to which it is antigenically highly related (Kapikian et al., Adv. Exp. Med. Biol. 327:59-69, 1992). Since there was a need for a multivalent vaccine that would induce resistance to each of the four major human rotavirus serotypes, the Jennerian

10   approach was modified by constructing three reassortant viruses using conventional genetic techniques of gene reassortment in tissue culture. Each single gene reassortant virus contained 10 RRV genes plus a single human rotavirus gene that coded for the major neutralization antigen (VP7) of serotype 1, 2, or 4. The intent was to prepare single gene substitution RRV reassortants with the attenuation characteristics of this simian

15   virus and the neutralization specificity of human rotavirus serotype 1, 2, or 4. The quadrivalent vaccine based on the host range restriction of the simian RRV in humans provided a high level of efficacy against human rotavirus infection in infants and young children (Perez-Schael et al., N. Engl. J. Med. 337:1181-1187, 1997). However, the vaccine virus retains mild reactogenicity in older seronegative infants lacking maternal

20   antibody, therefore a second generation Jennerian vaccine, based on the UK strain of bovine rotavirus, is being developed to replace the RRV vaccine (Clements-Mann et al., Vaccine 17:2715-2725, 1999).

The Jennerian approach also is being explored to develop vaccines for parainfluenza type 1 virus and for hepatitis A virus which are attenuated and

25   immunogenic in non-human primates (Emerson et al., J. Infect. Dis. 173:592-597, 1996; Hurwitz et al., Vaccine 15:533-540, 1997). The Jennerian approach was used for the development of a live attenuated vaccine for influenza A virus but it failed to produce a consistently attenuated vaccine for use in humans (Steinhoff et al., J. Infect. Dis. 163:1023-1028, 1991). As another example, reassortant viruses that contain two gene

30   segments encoding the hemagglutinin and neuraminidase surface glycoproteins from a human influenza A virus and the six remaining gene segments from an avian influenza A virus were attenuated in humans (Clements et al., J. Clin. Microbiol. 27:219-222, 1989; Murphy et al., J. Infect. Dis. 152:225-229, 1985; and Snyder et al., J. Clin. Microbiol. 23:852-857, 1986). This indicated that one or more of the six gene segments of the avian

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virus attenuated the avian-human influenza A viruses for humans. The genetic determinants of this attenuation were mapped using reassortant viruses possessing a single gene segment from an attenuating avian influenza A virus and the remaining genes from a human strain. It was shown that the nonstructural (NS), polymerase (PB1, PB2)

5      and M genes contributed to the attenuation phenotype of avian influenza A viruses in humans (Clements et al., J. Clin. Microbiol. 30:655-662, 1992).

In another study, the severe host range restriction of bovine respiratory syncytial virus (BRSV) for replication in chimpanzees was only slightly alleviated by replacement of the BRSV F and G glycoproteins with their HRSV counterparts. This

10     indicated that F and G are involved in this host range restriction, but that one or more additional bovine RSV genes are also involved (Buchholz et al., J. Virol. 74:1187-1199, 2000). This illustrates that more than one gene can contribute in unpredictable ways to the host range restriction phenotype of a mammalian or avian virus in primates.

The instant invention provides a new basis for attenuating a wild type or

15     mutant parental virus for use as a vaccine against HPIV, in which attenuation is based completely or in part on host range effects, while at least one or more of the major neutralization and protective antigenic determinant(s) of the chimeric virus is homologous to the virus against which the vaccine is directed. The HN and F proteins of BPIV3 are each approximately 80% related by amino acid sequence to their corresponding HPIV3

20     proteins (Suzu et al., Nucleic Acids Res. 15:2945-2958, 1987, incorporated herein by reference) and 25% related by antigenic analysis (Coelingh et al., J. Virol. 64:3833-3843, 1990; Coelingh et al., J. Virol. 60:90-96, 1986; van Wyke Coelingh et al., J. Infect. Dis. 157:655-662, 1988, each incorporated herein by reference). Previous studies indicated that two strains of BPIV3, the Kansas (Ka) strain and the Shipping Fever (SF) prototype

25     strain, were attenuated for the upper and lower respiratory tract of rhesus monkeys, and one of these, the Ka strain, was attenuated in chimpanzees (van Wyke Coelingh et al., 1988, supra, incorporated herein by reference). Immunization of nonhuman primates with the Ka virus induced antibodies reactive with HPIV3 and induced resistance to the replication of the human virus in the upper and the lower respiratory tract of monkeys

30     (id.) Subsequent evaluation of the Ka strain in humans indicated that the virus was satisfactorily attenuated for seronegative infants, and it retained the attenuation phenotype following replication in fully susceptible infants and children (Karron et al., 1996, supra; and Karron et al., 1995a, supra; each incorporated herein by reference). Its major advantages therefore were that it was satisfactorily attenuated for fully susceptible

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seronegative infants and children, and its attenuation phenotype was stable following replication in humans.

However, the level of serum hemagglutination-inhibiting antibodies reactive with HPIV3 induced in seronegative vaccinees who received $10^{5.0}$ tissue culture

5   infectious dose$_{50}$ (TCID)$_{50}$ of the Ka strain of BPIV3 was 1:10.5, which was three-fold lower than similar vaccinees who received a live attenuated HPIV3 vaccine (Karron et al., 1995a, *supra*; and Karron et al., 1995b, *supra*; each incorporated herein by reference). This lower level of antibodies to the human virus induced by BPIV3 reflected in large part the antigenic divergence between HPIV3 and BPIV3 (Karron et al., 1996, *supra*; and

10   Karron et al., 1995a, *supra*; each incorporated herein by reference). Studies to determine the efficacy of the Ka vaccine candidate against HPIV3 in humans have not been performed, but it is likely that this reduced level of antibodies reactive with HPIV3 will be reflected in a reduced level of protective efficacy.

Although it is clear that BPIV3 has host range genes that restrict

15   replication in the respiratory tract of rhesus monkeys, chimpanzees and humans, it remains unknown which of the bovine proteins or noncoding sequences contribute to this host range restriction of replication. It is possible that any of the BPIV3 proteins or noncoding sequences may confer a host range phenotype. It is not possible to determine in advance which genes or genome segments will confer an attenuation phenotype. This

20   can only be accomplished by systematic substitution of BPIV3 coding and non-coding sequences for their HPIV3 counterparts and by evaluation of the recovered HPIV3/BPIV3 chimeric viruses in seronegative rhesus monkeys or humans.

Despite the numerous advances toward development of effective vaccine agents against PIV serotypes 1, 2, and 3, there remains a clear need in the art for

25   additional tools and methods to engineer safe and effective vaccines to alleviate the serious health problems attributable to PIV, particularly illnesses among infants and children due to infection by HPIV. Among the remaining challenges in this context is the need for additional tools to generate suitably attenuated, immunogenic and genetically stable vaccine candidates for use in diverse clinical settings. To facilitate these goals,

30   existing methods for identifying and incorporating attenuating mutations into recombinant vaccine strains must be expanded. Furthermore, it is recognized that methods and compositions for designing vaccines against human PIV can be implemented as well to design novel vaccine candidates for veterinary use. Surprisingly,

the present invention fulfills these needs and provides additional advantages as described hereinbelow.

SUMMARY OF THE INVENTION

The present invention provides human-bovine chimeric parainfluenza viruses (PIVs) that are infectious and attenuated in humans and other mammals. In related aspects, the invention provides novel methods for designing and producing attenuated, human-bovine chimeric PIVs that are useful in various compositions to generate a desired immune response against PIV in a host susceptible to PIV infection. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a chimeric PIV genome or antigenome including a partial or complete human or bovine PIV "background" genome or antigenome combined or integrated with one or more heterologous gene(s) or genome segment(s) of a different PIV virus. Also provided within the invention are methods and compositions incorporating human-bovine chimeric PIV for prophylaxis and treatment of PIV infection.

The invention thus involves a method for developing live attenuated PIV vaccine candidates based on chimeras between HPIVs and BPIV3. Chimeras are generated using a cDNA-based virus recovery system. Recombinant viruses made from cDNA replicate independently and are propagated in the same manner as if they were biologically-derived viruses. Chimeric human-bovine PIV of the invention are recombinantly engineered to incorporate nucleotide sequences from both human and bovine PIV strains to produce an infectious, chimeric virus or subviral particle. In this manner, candidate vaccine viruses are recombinantly engineered to elicit an immune response against PIV in a mammalian host susceptible to PIV infection, including humans and non-human primates. Human-bovine chimeric PIV according to the invention may be engeneered to elicit an immune response to a specific PIV, e.g., HPIV3, or a polyspecific response against multiple PIVs, e.g., HPIV1 and HPIV3. Additional chimeric viruses can be designed in accordance with the teachings herein which serve as vectors for antigens of non-PIV pathogens, for example respiratory syncytial virus (RSV) or measles virus.

Exemplary human-bovine chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome comprising both human and bovine polynucleotide

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sequences, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants

5      or other additional components.

Chimeric human-bovine PIV of the invention include a partial or complete "background" PIV genome or antigenome derived from or patterned after a human or bovine PIV strain or subgroup virus combined with one or more heterologous gene(s) or genome segment(s) of a different PIV strain or subgroup virus to form the human-bovine

10     chimeric PIV genome or antigenome. In preferred aspects of the invention, chimeric PIV incorporate a partial or complete human PIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a bovine PIV.

The partial or complete background genome or antigenome typically acts

15     as a recipient backbone or vector into which are imported heterologous genes or genome segments of the counterpart, human or bovine PIV. Heterologous genes or genome segments from the counterpart, human or bovine PIV represent "donor" genes or polynucleotides that are combined with, or substituted within, the background genome or antigenome to yield a human-bovine chimeric PIV that exhibits novel phenotypic

20     characteristics compared to one or both of the contributing PIVs. For example, addition or substitution of heterologous genes or genome segments within a selected recipient PIV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity, or other desired phenotypic changes as compared with a corresponding phenotype(s) of the unmodified recipient and/or donor.

25            Genes and genome segments that may be selected for use as heterologous substitutions or additions within human-bovine chimeric PIV of the invention include genes or genome segments encoding a PIV N, P, C, D, V, M, F, SH (where appropriate), HN and/or L protein(s) or portion(s) thereof. In addition, genes and genome segments encoding non-PIV proteins, for example, an SH protein as found in mumps and SV5

30     viruses, may be incorporated within human-bovine PIV of the invention. Regulatory regions, such as the extragenic 3' leader or 5' trailer regions, and gene-start, gene-end, intergenic regions, or 3' or 5' non-coding regions, are also useful as heterologous substitutions or additions.

Preferred human-bovine chimeric PIV vaccine candidates of the invention

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bear one or more of the major antigenic determinants of HPIV3 in a background which is
attenuated by the substitution or addition of one or more BPIV3 genes or genome
segments.  The major protective antigens of PIVs are their HN and F glycoproteins,
although other proteins can also contribute to a protective immune response.  In certain

5       embodiments, the background genome or antigenome is an HPIV genome or antigenome,
e.g., an HPIV3, HPIV2, or HPIV1 background genome or antigenome, to which is added
or into which is substituted one or more BPIV gene(s) or genome segment(s), preferably
from BPIV3.  In one exemplary embodiment described below, an ORF of the N gene of a
BPIV3 is substituted for that of an HPIV.  Alternatively, the background genome or

10      antigenome may be a BPIV genome or antigenome which is combined with one or more
genes or genome segments encoding a HPIV3, HPIV2, or HPIV1 glycoprotein,
glycoprotein domain or other antigenic determinant.

In accordance with the methods of the invention, any BPIV gene or
genome segment, singly or in combination with one or more other BPIV genes, can be

15      combined with HPIV sequences to give rise to a human-bovine chimeric PIV vaccine
candidate.  Any HPIV, including different strains of a particular HPIV serotype, e.g.,
HPIV3 will be a reasonable acceptor for attenuating BPIV gene(s).  In general, the HPIV3
gene(s) or genome segment(s) selected for inclusion in a human-bovine chimeric PIV for
use as a vaccine against human PIV will include one or more of the HPIV protective

20      antigens such as the HN or F glycoproteins.

In exemplary aspects of the invention, human-bovine chimeric PIV
bearing one or more bovine gene(s) or genome segment(s) exhibits a high degree of host
range restriction, e.g., in the respiratory tract of mammalian models of human PIV
infection such as non-human primates.  In exemplary embodiments a human PIV is

25      attenuated by the addition or substitution of one or more bovine gene(s) or genome
segment(s) to a partial or complete human, e.g., HPIV3, PIV background genome or
antigenome.  In one example, the HPIV3 N gene is substituted by the BPIV3 N gene to
yield a novel human-bovine chimeric PIV vaccine candidate.

Preferably, the degree of host range restriction exhibited by human-bovine

30      chimeric PIV vaccine candidates of the invention is comparable to the degree of host
range restriction exhibited by the respective BPIV parent or "donor" strain.  Preferably,
the restriction should have a true host range phenotype, i.e., it should be specific to the
host in question and should not restrict replication and vaccine preparation *in vitro* in a
suitable cell line.  In addition, human-bovine chimeric PIV bearing one or more bovine

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gene(s) or genome segment(s) elicit a high level of resistance in hosts susceptible to PIV infection. Thus, the invention provides a new basis for attenuating a live virus vaccine against PIV, one which is based on host range effects due to the introduction of one or more gene(s) or genome segment(s) from a heterologous PIV, e.g., between HPIV3 and

5      BPIV3.

In related aspects of the invention, human-bovine chimeric PIV incorporates one or more heterologous gene(s) that encode an HPIV HN and/or F glycoprotein(s). Alternatively, the chimeric PIV may incorporate one or more genome segment(s) encoding an ectodomain (and alternatively a cytoplasmic domain and/or

10     transmembrane domain), or immunogenic epitope of an HPIV HN and/or F glycoprotein(s). These immunogenic proteins, domains and epitopes are particularly useful within human-bovine chimeric PIV because they generate novel immune responses in an immunized host. In particular, the HN and F proteins, and immunogenic domains and epitopes therein, provide major protective antigens.

15     In certain embodiments of the invention, addition or substitution of one or more immunogenic gene(s) or genome segment(s) from a human PIV subgroup or strain to or within a bovine background, or recipient, genome or antigenome yields a recombinant, chimeric virus or subviral particle capable of generating an immune response directed against the human donor virus, including one or more specific human

20     PIV subgroups or strains, while the bovine backbone confers an attenuated phenotype making the chimera a useful candidate for vaccine development. In one exemplary embodiment, one or more human PIV glycoprotein genes, e.g., HN and/or F, are added to or substituted within a partial or complete bovine genome or antigenome to yield an attenuated, infectious human-bovine chimera that elicits an anti-human PIV immune

25     response in a susceptible host.

In alternate embodiments, human-bovine chimeric PIV additionally incorporate a gene or genome segment encoding an immunogenic protein, protein domain or epitope from multiple human PIV strains, for example two HN or F proteins or immunogenic portions thereof each from a different HPIV, e.g., HPIV1 or HPIV2.

30     Alternatively, one glycoprotein or immunogenic determinant may be provided from a first HPIV, and a second glycoprotein or immunogenic determinant may be provided from a second HPIV by substitution without the addition of an extra glycoprotein- or determinant- encoding polynucleotide to the genome or antigenome. Substitution or addition of HPIV glycoproteins and antigenic determinants may also be achieved by

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construction of a genome or antigenome that encodes a chimeric glycoprotein in the recombinant virus or subviral particle, for example having an immunogenic epitope, antigenic region or complete ectodomain of a first HPIV fused to a cytoplasmic domain of a heterologous HPIV. For example, a heterologous genome segment encoding a

5        glycoprotein ectodomain from a HPIV1 or HPIV2 HN or F glycoprotein may be joined with a genome segment encoding a corresponding HPIV3 HN or F glycoprotein cytoplasmic/endodomain in the background genome or antigenome.

In alternate embodiments a human-bovine chimeric PIV genome or antigenome may encode a substitute, extra, or chimeric glycoprotein or antigenic

10       determinant thereof in the recombinant virus or subviral particle, to yield a viral recombinant having both human and bovine glycoproteins, glycoprotein domains, or immunogenic epitopes. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human PIV HN or F glycoprotein may be joined with a genome segment encoding a corresponding bovine HN or F glycoprotein

15       cytoplasmic/endodomain in the background genome or antigenome. Alternatively, the human PIV HN or F glycoprotein or parts thereof may be joined with a genome segment encoding an HN or F glycoprotein or parts thereof from another PIV strain or serotype.

Thus, according to the methods of the invention, human-bovine chimeric PIV may be constructed by substituting the heterologous gene or genome segment for a

20       counterpart gene or genome segment in a partial PIV background genome or antigenome. Alternatively, the heterologous gene or genome segment may be added as a supernumerary gene or genome segment in combination with a complete (or partial if another gene or genome segment is deleted) PIV background genome or antigenome. For example, two human PIV HN or F genes or genome segments can be included, one each

25       from HPIV2 and HPIV3.

Often, a heterologous gene or genome segment is added near an intergenic position within a partial or complete PIV background genome or antigenome. Alternatively, the gene or genome segment can be placed in other noncoding regions of the genome, for example, within the 5' or 3' noncoding regions or in other positions

30       where noncoding nucleotides occur within the partial or complete genome or antigenome. In one aspect, noncoding regulatory regions contain cis-acting signals required for efficient replication, transcription, and translation, and therefore represent target sites for modification of these functions by introducing a heterologous gene or genome segment or other mutation as disclosed herein.

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In more detailed aspects of the invention, attenuating mutations are introduced into cis-acting regulatory regions to yield, e.g., (1) a tissue specific attenuation (Gromeier et al., J. Virol. 73:958-964, 1999; Zimmermann et al., J. Virol. 71:4145-4149, 1997), (2) increased sensitivity to interferon (Zimmermann et al., 1997, *supra*), (3)

5      temperature sensitivity (Whitehead et al., 1998a, *supra*), (4) a general restriction in level of replication (Men et al., J. Virol. 70:3930-3937, 1996; Muster et al., Proc. Natl. Acad. Sci. USA 88:5177-5181, 1991), and/or (5) host specific restriction of replication (Cahour et al., Virology 207:68-76, 1995). These attenuating mutations can be achieved in various ways to produce an attenuated human-bovine chimeric PIV of the invention, for

10     example by point mutations, swaps of sequences between related viruses, or nucleotide deletions.

In yet additional alternative methods provided herein, a heterologous gene or genome segment may be added or substituted at a position corresponding to a wild-type gene order position of a counterpart gene or genome segment within the partial or

15     complete PIV background genome or antigenome. In other embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promotor-distal compared to a wild-type gene order position of a counterpart gene or genome segment within the background genome or antigenome, to enhance or reduce expression, respectively, of the heterologous gene or genome segment.

20     In general aspects of the invention, bovine genes or genome segments may be added to or substituted within a human PIV background to form an attenuated, human-bovine chimeric PIV. Alternatively, the chimera may be comprised of one or more human gene(s) or genome segment(s) added to or substituted within a bovine PIV background to form an attenuated PIV vaccine candidate. In this context, a chimeric PIV

25     genome or antigenome is formed of a partial or complete bovine PIV background genome or antigenome combined with a heterologous gene or genome segment from a human PIV. In preferred aspects, one or more bovine PIV gene(s) or genome segment(s) is substituted for a counterpart gene(s) or genome segment(s) within a human PIV background genome or antigenome. In alternate embodiments, one or more human PIV

30     glycoprotein genes, e.g., HN and/or F or a genome segment encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of a human PIV glycoprotein gene is substituted for a counterpart gene or genome segment within the bovine PIV background genome or antigenome. For example, both human PIV

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glycoprotein genes HN and F may be substituted to replace counterpart HN and F glycoprotein genes in a bovine PIV background genome or antigenome.

In a parallel fashion, the chimeric human-bovine PIV of the invention can be readily designed as "vectors" to incorporate antigenic determinants from different

5    pathogens, including more than one PIV strain or group (e.g., both human PIV3 and human PIV1), respiratory syncytial virus (RSV), measles and other pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference).

In more detailed aspects of the invention, human-bovine chimeric PIV are

10   comprised of a partial or complete BPIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV. Within these aspects, one or more of the HPIV glycoprotein genes HN and F, or one or more genome segments encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of the HN and/or F genes, may be added to a BPIV background

15   genome or antigenome or substituted for one or more counterpart genes or genome segments within the BPIV background genome or antigenome to yield the chimeric construct. Often, both HPIV glycoprotein genes HN and F will be substituted to replace counterpart HN and F glycoprotein genes in the BPIV background genome or antigenome, as exemplified by the recombinant chimeric virus $rBPIV3-F_HHN_H$ described

20   below. This is a desirable construct because it combines the antigenic determinants of the human PIV with the host range restricting elements of the bovine PIV.

In combination with the host range phenotypic effects provided in the human-bovine chimeric PIV of the invention, it is often desirable to adjust the attenuation phenotype by introducing additional mutations that increase or decrease attenuation of the

25   chimeric virus. Thus, in additional aspects of the invention, attenuated, human-bovine chimeric PIV are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations specifying an attenuating phenotype in the resultant virus or subviral particle. These can include mutations in RNA regulatory sequences or in encoded proteins. These attenuating mutations may be

30   generated *de novo* and tested for attenuating effects according to a rational design mutagenesis strategy. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant PIV and thereafter incorporated into a human-bovine chimeric PIV of the invention.

Introduction of attenuating and other desired phenotype-specifying mutations into chimeric bovine-human PIV of the invention may be achieved by transferring a heterologous gene or genome segment, e.g., a gene encoding an L protein or portion thereof, into a bovine or human PIV background genome or antigenome.

5 Alternatively, the mutation may be present in the selected background genome or antigenome, and the introduced heterologous gene or genome segment may bear no mutations or may bear one or more different mutations. Typically, the human or bovine background or "recipient" genome or antigenome is modified at one or more sites corresponding to a site of mutation in a heterologous virus (e.g., a heterologous bovine or

10 human PIV or a non-PIV negative stranded RNA virus) to contain or encode the same or a conservatively related mutation (e.g., a conservative amino acid substitution) as that identified in the donor virus (see, PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference). In one exemplary embodiment, a bovine background

15 or "recipient" genome or antigenome is modified at one or more sites corresponding to a site of mutation in HPIV3 JS cp45, as enumerated below, to contain or encode the same or a conservatively related mutation as that identified in the cp45 "donor."

Preferred mutant PIV strains for identifying and incorporating attenuating mutations into bovine-human chimeric PIV of the invention include cold passaged (cp),

20 cold adapted (ca), host range restricted (hr), small plaque (sp), and/or temperature sensitive (ts) mutants, for example the JS HPIV3 cp 45 mutant strain. In exemplary embodiments, one or more attenuating mutations occur in the polymerase L protein, e.g., at a position corresponding to $Tyr_{942}$, $Leu_{992}$, or $Thr_{1558}$ of JS wild type HPIV3. Alternatively, attenuating mutations in the N protein may be selected and incorporated in

25 a human-bovine chimeric PIV, for example which encode amino acid substitution(s) at a position corresponding to residues $Val_{96}$ or $Ser_{389}$ of JS. Alternative or additional mutations may encode amino acid substitution(s) in the C protein, e.g., at a position corresponding to $Ile_{96}$ of JS and in the M protein, e.g., at a position corresponding to $Pro_{199}$ (for example a $Pro_{199}$ to Thr mutation). Yet additional mutations for adjusting

30 attenuation of a human-bovine chimeric PIV of the invention are found in the F protein, e.g., at a position corresponding to $Ile_{420}$ or $Ala_{450}$ of JS, and in the HN protein, e.g., at a position corresponding to residue $Val_{384}$ of JS.

Attenuating mutations from biologically derived PIV mutants for incorporation into human-bovine chimeric PIV of the invention also include mutations in

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noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS cp45. Yet additional exemplary mutations may be engineered in the N gene start

5   sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS cp45.

From JS cp45 and other biologically derived PIV and non-PIV mutants, a large "menu" of attenuating mutations is provided, each of which mutations can be combined with any other mutation(s) for adjusting the level of attenuation in a

10  recombinant PIV bearing a genome or antigenome that is a chimera of human and bovine gene(s) or genome segment(s). For example, mutations within recombinant PIV of the invention include one or more, and preferably two or more, mutations of JS cp45. Desired human-bovine chimeric PIV of the invention selected for vaccine use often have at least two and sometimes three or more attenuating mutations to achieve a satisfactory

15  level of attenuation for broad clinical use. Preferably, recombinant human-bovine chimeric PIV incorporate one or more attenuating mutation(s) stabilized by multiple nucleotide substitutions in a codon specifying the mutation.

Additional mutations which can be adopted or transferred to human-bovine chimeric PIV of the invention may be identified in non-PIV nonsegmented

20  negative stranded RNA viruses and incorporated in PIV mutants of the invention. This is readily accomplished by mapping the mutation identified in a heterologous negative stranded RNA virus to a corresponding, homologous site in a recipient PIV genome or antigenome and mutating the existing sequence in the recipient to the mutant genotype (either by an identical or conservative mutation), as described in PCT/US00/09695 filed

25  April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference.

In addition to recombinant human-bovine chimeric PIV, the invention provides related cDNA clones, vectors and particles, each of which incorporate HPIV and BPIV sequences and, optionally, one or more of the additional, phenotype-specific

30  mutations set forth herein. These are introduced in selected combinations, e.g., into an isolated polynucleotide which is a recombinant cDNA genome or antigenome, to produce a suitably attenuated, infectious virus or subviral particle upon expression, according to the methods described herein. This process, coupled with routine phenotypic evaluation, provides human-bovine chimeric PIV having such desired characteristics as attenuation,

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temperature sensitivity, altered immunogenicity, cold-adaptation, small plaque size, host range restriction, genetic stability, etc. In particular, vaccine candidates are selected which are attenuated and yet are sufficiently immunogenic to elicit a protective immune response in the vaccinated mammalian host.

5          In yet additional aspects of the invention, human-bovine chimeric PIV, with or without additional mutations adopted, e.g., from a biologically derived attenuated mutant virus, are constructed to have additional nucleotide modification(s) to yield a desired phenotypic, structural, or functional change. Typically, the selected nucleotide modification will specify a phenotypic change, for example a change in growth

10         characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host range restriction, or immunogenicity. Structural changes in this context include introduction or ablation of restriction sites into PIV encoding cDNAs for ease of manipulation and identification.

          In preferred embodiments, nucleotide changes within the genome or

15         antigenome of a human-bovine chimeric PIV include modification of a viral gene by partial or complete deletion of the gene or reduction or ablation (knock-out) of its expression. These modifications can be introduced within the human or bovine background genome or antigenome, or may be introduced into the chimeric genome or antigenome by incorporation within the heterologous gene(s) or genome segment(s)

20         added or substituted therein. Target genes for mutation in this context include any of the PIV genes, including the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, small hydrophobic SH protein, where applicable, fusion protein F, and the products of the C, D and V open reading frames (ORFs). To the extent that the recombinant virus remains

25         viable and infectious, each of these proteins can be selectively deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to achieve novel deletion or knock out mutants. For example, one or more of the C, D, and/or V genes may be deleted in whole or in part, or its expression reduced or ablated (e.g., by introduction of a stop codon, by a mutation in an RNA editing site, by a mutation

30         that alters the amino acid specified by an initiation codon, or by a frame shift mutation in the targeted ORF(s). In one embodiment, a mutation can be made in the editing site that prevents editing and ablates expression of proteins whose mRNA is generated by RNA editing (Kato et al., EMBO J. 16:578-587, 1997a and Schneider et al., Virology 227:314-322, 1997, each incorporated herein by reference). Alternatively, one or more of the C,

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D, and/or V ORF(s) can be deleted in whole or in part to alter the phenotype of the resultant recombinant clone to improve growth, attenuation, immunogenicity or other desired phenotypic characteristics (see, U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, incorporated herein by reference).

5          Alternative nucleotide modifications in human-bovine chimeric PIV of the invention include a deletion, insertion, addition or rearrangement of a cis-acting regulatory sequence for a selected gene in the recombinant genome or antigenome. As with other such modifications described herein, these modifications can be introduced within the human or bovine background genome or antigenome, or may be introduced

10    into the chimeric genome or antigenome by incorporation within the heterologous gene(s) or genome segment(s) added or substituted therein. In one example, a cis-acting regulatory sequence of one PIV gene is changed to correspond to a heterologous regulatory sequence, which may be a counterpart cis-acting regulatory sequence of the same gene in a different PIV, or a cis-acting regulatory sequence of a different PIV gene.

15    For example, a gene end signal may be modified by conversion or substitution to a gene end signal of a different gene in the same PIV strain. In other embodiments, the nucleotide modification may comprise an insertion, deletion, substitution, or rearrangement of a translational start site within the recombinant genome or antigenome, e.g., to ablate an alternative translational start site for a selected form of a protein.

20          In addition, a variety of other genetic alterations can be produced in a human-bovine chimeric PIV genome or antigenome, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV. For example, genes or genome segments from non-PIV sources may be inserted in whole or in part. Alternatively, the order of genes can be changed, or a PIV genome promoter replaced

25    with its antigenome counterpart. Different or additional modifications in the recombinant genome or antigenome can be made to facilitate manipulations, such as the insertion of unique restriction sites in various non-coding regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

         In yet additional aspects, polynucleotide molecules or vectors encoding the

30    human-bovine chimeric PIV genome or antigenome can be modified to encode non-PIV sequences, e.g., a cytokine, a T-helper epitope, a restriction site marker, or a protein or immunogenic epitope of a microbial pathogen (e.g., virus, bacterium, parasite, or fungus) capable of eliciting a protective immune response in an intended host. In one such embodiment, human-bovine chimeric PIV are constructed that incorporate a gene or

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genome segment from a respiratory syncytial virus (RSV), for example a gene encoding an antigenic protein (e.g., an F or G protein), immunogenic domain or epitope of RSV.

In related aspects of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating a PIV-encoding cDNA) and methods are

5    provided for producing an isolated infectious human-bovine chimeric PIV. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a human-bovine chimeric PIV genome or antigenome. Also provided is the same or different expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins.

10   These proteins also can be expressed directly from the genome or antigenome cDNA. The vector(s) is/are preferably expressed or coexpressed in a cell or cell-free lysate, thereby producing an infectious human-bovine chimeric PIV viral particle or subviral particle.

The above methods and compositions for producing human-bovine

15   chimeric PIV yield infectious viral or subviral particles, or derivatives thereof. A recombinant infectious virus is comparable to the authentic PIV virus particle and is infectious as is. It can directly infect fresh cells. An infectious subviral particle typically is a subcomponent of the virus particle which can initiate an infection under appropriate conditions. For example, a nucleocapsid containing the genomic or antigenomic RNA

20   and the N, P, and L proteins is an example of a subviral particle which can initiate an infection if introduced into the cytoplasm of cells. Subviral particles provided within the invention include viral particles which lack one or more protein(s), protein segment(s), or other viral component(s) not essential for infectivity.

In other embodiments the invention provides a cell or cell-free lysate

25   containing an expression vector which comprises an isolated polynucleotide molecule comprising a human-bovine chimeric PIV genome or antigenome as described above, and an expression vector (the same or different vector) which comprises one or more isolated polynucleotide molecules encoding the N, P, and L proteins of PIV. One or more of these proteins also can be expressed from the genome or antigenome cDNA. Upon expression

30   the genome or antigenome and N, P and L combine to produce an infectious human-bovine chimeric PIV virus or subviral particle.

The human-bovine chimeric PIVs of the invention are useful in various compositions to generate a desired immune response against PIV in a host susceptible to PIV infection. Human-bovine chimeric PIV recombinants are capable of eliciting a

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protective immune response in an infected mammalian host, yet are sufficiently
attenuated so as not to cause unacceptable symptoms of severe respiratory disease in the
immunized host.  In addition, the human-bovine chimeric PIV recombinants should
replicate with sufficient efficiency *in vitro* to make vaccine preparation feasible.  The

5      attenuated virus or subviral particle may be present in a cell culture supernatant, isolated
from the culture, or partially or completely purified.  The virus may also be lyophilized,
and can be combined with a variety of other components for storage or delivery to a host,
as desired.

                    The invention further provides novel vaccines comprising a

10     physiologically acceptable carrier and/or adjuvant and an isolated attenuated human-
bovine chimeric PIV virus or subviral particle.  In preferred embodiments, the vaccine is
comprised of a human-bovine chimeric PIV having at least one, and preferably two or
more additional mutations or other nucleotide modifications as described above to
achieve a suitable balance of attenuation and immunogenicity.  The vaccine can be

15     formulated in a dose of $10^3$ to $10^7$ PFU of attenuated virus.  The vaccine may comprise
attenuated human-bovine chimeric PIV that elicits an immune response against a single
PIV strain or against multiple PIV strains or groups.  In this regard, human-bovine
chimeric PIV can be combined in vaccine formulations with other PIV vaccine strains, or
with other viral vaccine viruses such as RSV.

20                     In related aspects, the invention provides a method for stimulating the
immune system to elicit an immune response against PIV in a mammalian subject.  The
method comprises administering a formulation of an immunologically sufficient amount
of a human-bovine chimeric PIV in a physiologically acceptable carrier and/or adjuvant.
In one embodiment, the immunogenic composition is a vaccine comprised of a human-

25     bovine chimeric PIV having at least one, and preferably two or more attenuating
mutations or other nucleotide modifications specifying a desired phenotype as described
above.  The vaccine can be formulated in a dose of $10^3$ to $10^7$ PFU of attenuated virus.
The vaccine may comprise attenuated human-bovine chimeric PIV virus that elicits an
immune response against a single PIV, against multiple PIVs, e.g., HPIV1 and HPIV3, or

30     against one or more PIV(s) and a non-PIV pathogen such as RSV.  In this context,
human-bovine chimeric PIV can elicit a monospecific immune response or a polyspecific
immune response against multiple PIVs, or against one or more PIV(s) and a non-PIV
pathogen such as RSV.  Alternatively, human-bovine chimeric PIV having different
immunogenic characteristics can be combined in a vaccine mixture or administered

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separately in a coordinated treatment protocol to elicit more effective protection against one PIV, against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen such as RSV. Preferably the immunogenic composition is administered to the upper respiratory tract, e.g., by spray, droplet or aerosol.

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## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates cloning of the N coding region of bovine PIV strains Ka or SF into HPIV3. In Fig. 1A-C, the BPIV3 N open reading frame (ORF) replaces its

10 corresponding HPIV3 sequence in the full-length rJS antigenomic cDNA (Durbin et al., 1997a, *supra*). BPIV3 Ka and SF N genes were first amplified by RT-PCR using standard molecular biological techniques from virion RNA and subcloned as 1.9 kb fragments into pBluescript to give pBS-KaN or pBS-SFN, respectively. The HPIV3 rJS N gene was subcloned as a 1.9 kb MluI/EcoRI fragment into pUC 119 from a plasmid

15 containing the 5' half of the rJS HPIV3 antigenome (Durbin et al., 1997a, *supra*; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference) to give pUC119JSN. Each N

20 gene was modified by site-directed mutagenesis to place an NcoI and AflII site at the translational start and stop sites, respectively. The Ka and SF N genes are identical in the translational start and stop site regions and, therefore, identical mutagenesis reactions were performed on both BPIV3 N genes as depicted in 1A. Fig. 1B--Following AflII/NcoI digestion, a 1.5 kb fragment from pBS-KaN or pBS-SFN representing the

25 BPIV3 N coding region was introduced into the NcoI/AflII window of the HPIV3 N subclone pUC119JSN-NcoI/AflII as a replacement for its HPIV3 counterpart. Fig. 1C-- Each chimeric subclone was then subjected to site-directed mutagenesis to restore the sequence present in HPIV3 rJS before the translation start codon or after the stop codon and BPIV3 coding sequence immediately after the start codon and before the stop codon.

30 This yielded pUC119B/HKaN and pUC119B/HSFN, which were used to import the BPIV3 N gene into the HPIV3 cDNA clone as shown in Fig. 2. Figure 1, Panel A, CAAAAATGTTG (SEQ ID NO. 10); GCAACTAATCGA (SEQ ID NO. 11); TAACCATGGTGA (SEQ ID NO. 12); GCACTTAAGCAC (SEQ ID NO. 13). Figure 1,

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Panel C, TAACCATGGTGA (SEQ ID NO. 12); GCACTTAAGCAC (SEQ ID NO. 13);

CAAAAATGTTGA (SEQ ID NO. 14); GCAACTAGTCGA (SEQ ID NO. 15).

Figure 2 illustrates insertion of the HPIV3/BPIV3 (strain Ka or SF)

chimeric N gene into the HPIV3 antigenomic cDNA. In Fig. 2A, the BPIV3 N ORF of

5      Ka or SF flanked by HPIV3 sequence was subcloned as an MluI/EcoRI fragment from

pUC119B/HKaN or pUC119B/HSFN and inserted into pLeft+2G (Durbin et al., 1997a,

*supra*). The pLeft+2G plasmid contains the 5' half of the HPIV3 rJS antigenome from nt

1-7437 (genome sense) behind a T7 promoter. The location of two G residues that were

inserted between T7 promoter and HPIV3 sequence to improve transcription is indicated

10     by an asterisk. Fig. 2B--An XhoI/NgoMI fragment of pRight (Durbin et al., 1997a, *supra*;

U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional

Application No. 60/047,575, filed May 23, 1997 (corresponding to International

Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed

September 19, 1997, each incorporated herein by reference) containing the 3' end of the

15     HPIV3 antigenome flanked by the hepatitis delta virus ribozyme and T7 terminator was

cloned into the XhoI/NgoMI window of the modified pLeft plasmid resulting in plasmids

pB/HPIV3KaN and pB/HPIV3SFN. Each of these chimeric constructs contains the

complete positive sense sequence of the HPIV3 antigenomic RNA except for the N

coding region which has been replaced by its BPIV3 Ka or SF counterpart.

20             Figure 3 provides nucleotide sequences of HPIV3, BPIV3 and chimeric

viruses of the invention around N translation start (A) and stop (B) codons. The position

of the individual ORFs is described in the respective Genbank reports (#AF178654 for

BPIV3 Ka, #AF178655 for BPIV3 SF and #Z11515) and included herein by reference.

The sequences (positive-sense) flanking the translational start (A) and stop (B) codons

25     (each underlined) in the N gene are shown for the parental recombinant HPIV3 JS (rJS),

the parental biologically-derived BPIV3 Ka and SF viruses (Ka and SF), and the chimeric

cKa and cSF viruses. Host-specific residues in the cKa and cSF virus sequences and their

counterparts in rJS (before the start codon and after the stop codon) and SF or Ka (start

codon through stop codon, inclusive) are in boldface type. Plaque-purified chimeric virus

30     was amplified by RT-PCR from virion RNA and sequenced using the Taq Dye Deoxy

Terminator Cycle kit (ABI, Foster City, CA). This confirmed that the predicted

sequences were present in each chimeric virus. Figure 3A. rJS,

GGAACTCTATAATTTCAAAAATGTTGAGCCTATTTGATAC (SEQ ID NO. 16).

Figure 3A. cKa and cSF,

GGAACTCTATAATTTCAAAAATGTTGAGTCTATTCGACAC (SEQ ID NO. 17).
Figure 3A. Ka and SF,
GAAATCCTAAGACTGTAATCATGTTGAGTCTATTCGACAC (SEQ ID NO. 18).
Figure 3B. rJS, TTAACGCATTTGGAAGCAACTAATCGAATCAACATTTTAA (SEQ
ID NO. 19).  Figure 3B. cKa and cSF,
TCAGTGCATTCGGAAGCAACTAGTCGAATCAACATTTTAA (SEQ ID NO. 20).
Figure 3B. Ka and SF,
TCAGTGCATTCGGAAGCAACTAGTCACAAAGAGATGACCA (SEQ ID NO. 21).

Figure 4 details the structure of the BPIV3/HPIV3 chimeric viruses of the
invention, and their confirmation by TaqI digestion of RT-PCR products generated from
virus RNA.  In Fig. 4A the genomes of the chimeric cKa and cSF viruses are shown
schematically (not to scale) relative to that of HPIV3 and BPIV3 parent viruses.  Ka- and
SF-specific regions are indicated by light and dark shading respectively.  Arrows above
the rJS genome indicate the locations of primers used for RT-PCR amplification of
chimeric and parent viruses for the purposes of diagnostic TaqI digestion.  These primers
were directed to regions conserved between HPIV3 and BPIV3 so that they could be used
for the amplification of HPIV3, BPIV3 and chimeric BPIV3/HPIV3 viruses.  In Fig. 4B
the expected sizes of TaqI digestion products for each virus are shown for a 1898-bp PCR
product amplified from RNA with the primer pair illustrated in Fig..4A.  This PCR
product is illustrated at the top in Fig. 4B, and the N ORF is indicated as a filled
rectangle.  TaqI fragments unique to each virus and which therefore serve in virus
identification are indicated with an asterisk. Fig. 4C provides TaqI profiles of PCR
products containing the PIV3 N coding region of chimeric cKa (left) or cSF (right)
flanked by those of the HPIV3 and BPIV3 parent viruses.  Unique TaqI fragments
diagnostic of virus identity and corresponding to those identified in (4B) are indicated
with an asterisk.  Calculated lengths (bp) of DNA gel bands are indicated.

Figure 5 provides multicycle growth curves of parental and chimeric
viruses in MDBK (A) or LLC-MK2 (B) cells.  Monolayers of bovine MDBK (A) or
simian LLC-MK2 (B) cells in wells (9.6 cm$^2$ each) of a 6 well plate were infected
individually at a multiplicity of infection of 0.01 with the indicated parental or chimeric
virus.  Three replicate infections were performed for each virus.  Samples were taken at
the indicated time points, stored at -70°C, and titered by TCID$_{50}$ assay in parallel.
Growth curves are constructed using the average of 3 replicate samples at each time

point. The lower limit of virus detectability was $10^{1.5}$TCID$_{50}$/ml, which is indicated by a dotted line.

Figures 6A-6G set forth the complete positive sense nucleotide sequence (SEQ ID NO. 22) of the bovine PIV3 Ka strain. .

5      Figures 7A-7G set forth the complete positive sense nucleotide sequence (SEQ ID NO. 23) of the bovine PIV3 SF strain.

Figure 8A provides a schematic depiction of the genomes of chimeric rHPIV3-F$_B$HN$_B$ and rBPIV3-F$_H$HN$_H$ viruses, and of their parent viruses, rHPIV3 JS and BPIV3 Ka (not to scale). The F and HN genes were exchanged in a single restriction

10     fragment between rHPIV3 and rBPIV3 using SgrAI and BsiWI sites that had been introduced in front of the M and HN gene end sequences, respectively.

Figure 8B depicts assembly of an antigenomic cDNA for BPIV3 Ka. A full length cDNA was constructed to encode the complete antigenomic sequence of BPIV3 Ka (GenBank accession #AF178654). The cDNA was assembled from subclones

15     derived from reverse transcription (RT) of viral (v)RNA and polymerase chain reaction (PCR) amplification. Multiple subclones of the antigenome were sequenced, and only clones matching the consensus sequence of BPIV3 Ka were used for assembly of the full length clone, with the exception of nt 21 and nt 23, which differ from the published sequence but occur with similar frequency in the virus population..

20     Figure 8C illustrates features of parental and chimeric bovine-human PIV genomes. The genomes of the chimeric rHPIV3 F$_B$HN$_B$ and rBPIV3 F$_H$HN$_H$ viruses and those of their parent viruses rHPIV3 JS and BPIV3 Ka are shown schematically (not to scale). Two unique restriction enzyme recognition sites, SgrAI and BsiWI, were introduced near the M and HN gene ends, respectively. The recombinant HPIV3 and

25     BPIV3 viruses bearing these introduced restriction sites were designeated rHPIV3s and rBPIV3s as indicated in Fugure 8C2. Glycoprotein genes were exchanged between rHPIV3 JS and rBPIV3 Ka. The nucleotide sequence that was mutagenized is shown below each cDNA construct, with the position of the first nucleotide of each sequence indicated. The introduced SgrAI and BsiWI restriction sites are underlined and

30     nucleotides that differ between HPIV3 and BPIV3 and thus identify the origin of the gene inserts are depicted in bold print. Figure 8C, Panel 1, rHPIV 3 JS, TCCACCGGTGCA (SEQ ID NO. 4), TAGACAAAAGGG (SEQ ID NO. 24). Figure 8C, Panel 1. rBPIV3 Ka, TCCAACATTGCA (SEQ ID NO. 2); AAGATATAAAGA (SEQ ID NO. 25). Figure 8C, Panel 2 rHPIV3s, CGCACCGGTGTA (SEQ ID NO. 5); TAGACGTACGGG

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(SEQ ID NO. 26). Figure 8C, Panel 2. rBPIV3s, TCCACCGGTGCA (SEQ ID NO. 3);

AAGACGTACGGA (SEQ ID NO. 27). Figure 8C, Panel 3. rHPIV3 $F_B HN_B$,

CGCACCGGTGCA (SEQ ID NO. 28); AAGACGTACGGG (SEQ ID NO. 29). Figure

8C, Panel 3. rBPIV3 $F_H HN_H$, AAGACGTACGGG (SEQ ID NO. 30);

5      TAGACGTACGGA (SEQ ID NO. 31).

Figure 9 provides a confirmation of the identity of recombinant viruses by

RT-PCR of viral RNA and *Eco* RI digestion. RT-PCR products of viral RNA were

prepared with a primer pair that recognized conserved regions on either side of the F and

HN genes in both BPIV3 and HPIV3. Digestion with *Eco* RI resulted in a unique pattern

10     of restriction fragments for each of the four viruses. In the schematic diagram on the left,

horizontal lines symbolize the amplified viral sequences and vertical bars show the

positions of *Eco* RI sites. The expected size of each restriction fragment is indicated

above the line. The numbers below each line correspond to the sequence position in the

antigenomic RNA of BPIV3 Ka, HPIV3 JS (GenBank accession #AF178654 and

15     Z11575), or of the indicated chimeric derivative. On the right, a 1% agarose gel of the

*Eco* RI digestion of PCR products is shown, confirming the identity of parental and

chimeric viruses. The asterisks indicate gel bands that contain two restriction fragments

that comigrate due to close similarity in size.

Figure 10 depicts multicycle replication of chimeric and parental viruses in

20     simian LLC-MK2 cells. Multicycle replication (the input inoculum had an MOI of 0.01)

of the three chimeric viruses rHPIV3-$F_B HN_B$, rBPIV3-$F_H HN_H$ and rHPIV3-$N_B$ (also

referred to as cKa) is compared with the replication of their parental viruses BPIV3 Ka

and rHPIV3. The virus titers are shown as mean $log_{10}$ TCID$_{50}$/ml ± standard error of

triplicate samples. The lower limit of detection of this assay is 10 TCID$_{50}$, as indicated by

25     the dotted horizontal line.

Figure 11 documents mean titers of chimeric and parental viruses in

nasopharyngeal swabs of infected rhesus monkeys over the course of infection. Virus

titers are shown as mean TCID$_{50}$/ml in LLC-MK2 cells ± standard error for groups of 4 or

6 monkeys infected with the same virus. This illustrates the same experiment as shown in

30     Table 3. In panel A, mean titers of rHPIV3-$F_B HN_B$ are compared to rHPIV3 and BPIV3

Ka titers. In panel B, mean rBPIV3-$F_H HN_H$ titers are compared to those of BPIV3 Ka

and rHPIV3, which, for the last two viruses, are the same values in panel A but are

presented separately to facilitate comparison. Day 5 titers were excluded from the figures

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because they were much lower than day 4 and day 6 titers, most likely due to technical problems during the sample collection.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

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The present invention provides recombinant parainfluenza virus (PIV) cloned as a chimera of human and bovine PIV genomic or antigenomic sequences to yield a human-bovine chimeric PIV. The chimeric construction of human-bovine PIV yields a viral particle or subviral particle that is infectious in mammals, particularly humans, and

10    useful for generating immunogenic compositions for clinical or veterinary use. Also provided within the invention are novel methods and compositions for designing and producing attenuated, human-bovine chimeric PIV, as well as methods and compositions for the prophylaxis and treatment of PIV infection.

Chimeric human-bovine PIV of the invention are recombinantly

15    engineered to incorporate nucleotide sequences from both human and bovine PIV strains to produce an infectious, chimeric virus or subviral particle. In this manner, candidate vaccine viruses are recombinantly engineered to elicit an immune response against PIV in a mammalian host susceptible to PIV infection, including humans and non-human primates. Human-bovine chimeric PIV according to the invention may elicit an immune

20    response to a specific PIV, e.g., HPIV3, or a polyspecific response against multiple PIVs, e.g., HPIV1 and HPIV3.

Exemplary human-bovine chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome comprising both human and bovine polynucleotide sequences, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein

25    (P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components.

Chimeric human-bovine PIV of the invention include a partial or complete

30    "background" PIV genome or antigenome derived from or patterned after a human or bovine PIV strain or serotype virus combined with one or more heterologous gene(s) or genome segment(s) of a different PIV strain or serotype virus to form the human-bovine chimeric PIV genome or antigenome. In certain aspects of the invention, chimeric PIV incorporate a partial or complete human PIV (HPIV) background genome or antigenome

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combined with one or more heterologous gene(s) or genome segment(s) from a bovine PIV. In alternate aspects of the invention, chimeric PIV incorporate a partial or complete bovine PIV (BPIV) background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV.

5         The partial or complete background genome or antigenome typically acts as a recipient backbone or vector into which are imported heterologous genes or genome segments of the counterpart, human or bovine PIV. Heterologous genes or genome segments from the counterpart, human or bovine PIV represent "donor" genes or polynucleotides that are combined with, or substituted within, the background genome or

10    antigenome to yield a human-bovine chimeric PIV that exhibits novel phenotypic characteristics compared to one or both of the contributing PIVs. For example, addition or substitution of heterologous genes or genome segments within a selected recipient PIV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity, or other desired phenotypic changes as compared with a corresponding

15    phenotype(s) of the unmodified recipient and/or donor. Genes and genome segments that may be selected for use as heterologous inserts or additions within human-bovine chimeric PIV of the invention include genes or genome segments encoding a PIV N, P, C, D, V, M, SH, where applicable, F, HN and/or L protein(s) or portion(s) thereof. Regulatory regions, such as the extragenic leader or trailer or intergenic regions, are also

20    useful as heterologous inserts or additions.

         The heterologous gene(s) or genome segment(s) may be added or substituted at a position corresponding to a wild-type gene order position of the counterpart gene(s) or genome segment(s) within the partial or complete PIV background genome or antigenome, which counterpart gene or genome segment is thereby replaced or displaced (e.g., to a more promotor-distal position). In yet additional embodiments, the

25    heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promotor-distal compared to a wild-type gene order position of the counterpart gene or genome segment within the background genome or antigenome, which enhances or reduces, respectively, expression of the heterologous gene or genome

30    segment.

         The introduction of heterologous immunogenic proteins, domains and epitopes to produce human-bovine chimeric PIV is particularly useful to generate novel immune responses in an immunized host. Addition or substitution of an immunogenic gene or genome segment from one, donor PIV within a recipient genome or antigenome

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of a different PIV can generate an immune response directed against the donor subgroup or strain, the recipient subgroup or strain, or against both the donor and recipient subgroup or strain. To achieve this purpose, human-bovine chimeric PIV may also be constructed that express a chimeric protein, e.g., an immunogenic glycoprotein having a

5   cytoplasmic tail and/or transmembrane domain specific to one PIV fused to an ectodomain of a different PIV to provide, e.g., a human-bovine fusion protein, or a fusion protein incorporating domains from two different human PIVs. In a preferred embodiment, a human-bovine chimeric PIV genome or antigenome encodes a chimeric glycoprotein in the recombinant virus or subviral particle having both human and bovine

10  glycoprotein domains or immunogenic epitopes. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human PIV HN or F glycoprotein may be joined with a polynucleotide sequence (i.e., a genome segment) encoding the corresponding bovine HN or F glycoprotein cytoplasmic and transmembrane domains to form the human-bovine chimeric PIV genome or antigenome.

15          In other embodiments, human-bovine chimeric PIV useful in a vaccine formulation can be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the HN and/or F proteins. This might involve the introduction of one or more point mutations; it might also involve an entire HN or F gene, or a genome segment encoding a particular immunogenic region thereof, from one

20  PIV strain or group is incorporated into a chimeric PIV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different PIV strain or group, or by adding one or more copies of the gene, such that multiple antigenic forms are represented. Progeny virus produced from the modified PIV clone can then be used in vaccination protocols against emerging PIV strains.

25          Replacement of a human PIV coding sequence or non-coding sequence (e.g., a promoter, gene-end, gene-start, intergenic or other cis-acting element) with a heterologous counterpart yields chimeric PIV having a variety of possible attenuating and other phenotypic effects. In particular, host range and other desired effects arise from substituting a bovine or murine PIV (MPIV) protein, protein domain, gene or genome

30  segment imported within a human PIV background, wherein the bovine or murine gene does not function efficiently in a human cell, e.g., from incompatibility of the heterologous sequence or protein with a biologically interactive human PIV sequence or protein (i.e., a sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.) or, more typically

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in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, bovine PIV sequences are selected for introduction into human PIV based on known aspects of bovine and human PIV structure and function.

5　　　　　　HPIV3 is a member of the *Respirovirus* genus of the *Paramyxoviridae* family in the order *Mononegavirales* (Collins et al., 1996, *supra*). HPIV3 is the best characterized of the HPIVs and represents the prototype HPIV. Its genome is a single strand of negative-sense RNA 15462 nucleotides (nt) in length (Galinski et al., Virology 165:499-510, 1988; and Stokes et al., Virus Res. 25:91-103, 1992; each incorporated

10　herein by reference). At least eight proteins are encoded by the PIV3 genome: the nucleocapsid protein N, the phosphoprotein P, the C and D proteins of unknown functions, the matrix protein M, the fusion glycoprotein F, the hemagglutinin-neuraminidase glycoprotein HN, and the large polymerase protein L (Collins et al., 1996, *supra*). A protein containing the V ORF in the P gene might also be produced (Durbin et

15　al., Virology 261:319-333, 1999)

　　　　　　The M, HN, and F proteins are envelope-associated, and the latter two are surface glycoproteins which, as is the case with each PIV, are the major neutralization and protective antigens (Collins et al., 1996, *supra*). The significant sequence divergence between comparable PIV HN or F proteins among the PIVs is thought to be the basis for

20　the type specificity of the protective immunity (Collins et al., 1996, *supra*; Cook et al., Amer. Jour. Hyg. 77:150-159, 1963; Ray et al., J. Infect. Dis. 162:746-749, 1990; each incorporated herein by reference).

　　　　　　The HPIV3 genes are each transcribed as a single mRNA that encodes a single protein, with the exception of the P mRNA which contains four ORFs, namely P,

25　C, D and V (Galinski et al., Virology 186:543-550, 1992; and Spriggs et al., J. Gen. Virol. 67:2705-2719, 1986; each incorporated herein by reference). The P and C proteins are translated from separate, overlapping ORFs in the mRNA. Whereas all paramyxoviruses encode a P protein, only members of the genus *Respirovirus* and *Morbillivirus* encode a C protein. Individual viruses vary in the number of proteins expressed from the C ORF and

30　in its importance in replication of the virus *in vitro* and *in vivo*. Sendai virus (SeV) expresses four independently initiated proteins from the C ORF: C', C, Y1, and Y2, whose translational start sites appear in that order in the mRNA (Curran, et al., Enzyme 44:244-249, 1990; Lamb et al., in The Paramyxoviruses, D. Kingsbury, ed., pp. 181-214, Plenum Press, New York, 1991; incorporated herein by reference), whereas HPIV3 and

measles virus (MeV) express only a single C protein (Bellini et al., J. Virol. 53:908-919, 1985; Sanchez et al., Virology 147:177-86, 1985; and Spriggs et al., 1986, *supra*; each incorporated herein by reference).

5          The PIV3 D protein is a fusion protein of the P and D ORFs, and is expressed from the P gene by the process of co-transcriptional RNA editing in which two nontemplated G residues are added to the P mRNA at the RNA editing site (Galinski et al., 1992, *supra*; and Pelet et al., EMBO J. 10:443-448, 1991; each incorporated herein by reference). BPIV3, the only other paramyxovirus which expresses a D protein, uses RNA editing to express this protein as well as a second protein, the V protein.

10         Nearly all members of the genera *Respirovirus*, *Rubulavirus*, and *Morbillivirus* express a V protein. The one member which clearly does not is HPIV1, which lacks an intact V ORF (Matsuoka et al., J. Virol. 65:3406-3410, 1991, incorporated herein by reference). The V ORF is characterized by the presence of a cysteine-rich domain that is highly conserved (Cattaneo et al., Cell 56:759-764, 1989; Park et al., J. 15 Virol. 66:7033-7039, 1992; Thomas et al., Cell 54:891-902, 1988; and Vidal et al., J. Virol. 64:239-246, 1990; each incorporated herein by reference). The V ORF is maintained in each of the HPIV3 viruses sequenced to date suggesting that this ORF is expressed and retains function for this virus (Galinski et al., Virology 155:46-60, 1986; Spriggs et al., 1986, *supra*; and Stokes et al., 1992, *supra*; incorporated herein by 20 reference).

           The BPIV3 V protein is expressed when one nontemplated G residue is added at the RNA editing site (Pelet et al., 1991, *supra*; incorporated herein by reference). However, in the case of HPIV3, two translation stop codons lie between the editing site and the V ORF, and it is not clear whether HPIV3 represents another example in which 25 this ORF is not expressed, or whether it is expressed by some other mechanism. One possibility is that HPIV3 editing also occurs at a second, downstream site in the P gene, although this did not appear to occur in cell culture (Galinski et al., 1992, *supra*). Alternatively, it might be that ribosomes gain access to the V ORF by ribosomal frameshifting. This would be comparable to the situation with the P locus of MV. MV 30 expresses C, P, and V proteins, but also expresses a novel R protein which is synthesized by frameshifting from the P ORF to the V ORF (Liston et al., J. Virol. 69:6742-6750, 1995, incorporated herein by reference). Genetic evidence suggests that the V ORF of HPIV3 is functional (Durbin et al., 1999, *supra*).

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Although the means by which HPIV3 expresses its V protein is unclear, the extreme conservation of the its V ORF in different strains suggests that it is indeed expressed. The function of the V protein is not well defined, but V-minus MV and SeV recombinants have been recovered that replicate efficiently *in vitro* but exhibit reduced

5       replication *in vivo* (Delenda, et al., Virology 228:55-62, 1997; Delenda et al., Virology 242:327-337, 1998; Kato et al., 1997a, *supra*; Kato et al., J. Virol. 71:7266-7272, 1997b; and Valsamakis et al., J. Virol. 72:7754-7761, 1998; each incorporated herein by reference).

The viral genome of PIV also contains extragenic leader and trailer

10      regions, possessing all or part of the promoters required for viral replication and transcription, as well as non-coding and intergenic regions. Thus, the PIV genetic map is represented as 3' leader-N-P/C/D/V-M-F-HN-L-5' trailer. Some viruses, such as simian virus 5 and mumps virus, have a gene located between F and HN that encodes a small hydrophobic (SH) protein of unknown function. Transcription initiates at the 3' end and

15      proceeds by a sequential stop-start mechanism that is guided by short conserved motifs found at the gene boundaries. The upstream end of each gene contains a gene-start (GS) signal, which directs initiation of its respective mRNA. The downstream terminus of each gene contains a gene-end (GE) motif which directs polyadenylation and termination. Exemplary sequences have been described for the human PIV3 strains JS (GenBank

20      accession number Z11575, incorporated herein by reference) and Washington (Galinski M.S., in The Paramyxoviruses, Kingsbury, D.W., ed., pp. 537-568, Plenum Press, New York, 1991, incorporated herein by reference), and for the bovine PIV3 strain 910N (GenBank accession number D80487, incorporated herein by reference).

As used herein, "PIV gene" generally refers to a portion of the PIV

25      genome encoding an mRNA and typically begins at the upstream end with a gene-start (GS) signal and ends at the downstream end with the gene-end (GE) signal. The term PIV gene also includes what is described as "translational open reading frame", or ORF, particularly in the case where a protein, such as C, is expressed from an additional ORF rather than from a unique mRNA. To construct human-bovine chimeric PIV of the

30      invention, one or more PIV gene(s) or genome segment(s) may be deleted, inserted or substituted in whole or in part. This means that partial or complete deletions, insertions and substitutions may include open reading frames and/or cis-acting regulatory sequences of any one or more of the PIV genes or genome segments. By "genome segment" is

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meant any length of continuous nucleotides from the PIV genome, which might be part of an ORF, a gene, or an extragenic region, or a combination thereof.

The instant invention involves a method for developing live attenuated PIV vaccine candidates based on chimeras between HPIVs and BPIV3. Chimeras are
5    constructed through a cDNA-based virus recovery system. Recombinant viruses made from cDNA replicate independently and are propagated in the same manner as if they were biologically-derived viruses. Preferred human-bovine chimeric PIV vaccine candidates of the invention bear one or more of the major antigenic determinants of one or more human PIV(s), e.g., HPIV1, HPIV2, and/or HPIV3, in a background which is
10   attenuated by the substitution or addition of one or more BPIV genes or genome segments. The major protective antigens of PIVs are their HN and F glycoproteins, although other proteins can also contribute to a protective immune response.

Thus, the invention provides a new basis for attenuating a wild type or mutant parental virus for use as a vaccine against PIV, one which is based on host range
15   effects due to the introduction of one or more gene(s) or genome segment(s) between HPIV and BPIV. There are numerous nucleotide and amino acid sequence differences between BPIV and HPIV, which are reflected in host range differences. For example, between HPIV3 and BPIV3 the percent amino acid identity for each of the following proteins is: N (86%), P (65%), M (93%), F (83%), HN (77%), and L (91%). The host
20   range difference is exemplified by the highly permissive growth of HPIV3 in rhesus monkeys, compared to the restricted replication of two different strains of BPIV3 in the same animal (van Wyke Coelingh et al., 1988, *supra*). Although the basis of the host range differences between HPIV3 and BPIV3 remains to be determined, it is likely that they will involve more than one gene and multiple amino acid differences. The
25   involvement of multiple genes and possibly cis-acting regulatory sequences, each involving multiple amino acid or nucleotide differences, gives a very broad basis for attenuation, one which cannot readily be altered by reversion. This is in contrast to the situation with other live attenuated HPIV3 viruses which are attenuated by one or several point mutations. In this case, reversion of any individual mutation may yield a significant
30   reacquisition of virulence or, in a case where only a single residue specified attenuation, complete reacquisition of virulence.

In exemplary embodiments of the invention described hereinbelow, the background genome or antigenome is an HPIV3 genome or antigenome, and the heterologous gene or genome segment is a N ORF derived from, alternatively, a Ka or SF

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strain of BPIV3 (which are 99% related in amino acid sequence). The N ORF of the
HPIV3 background antigenome is substituted by the counterpart BPIV3 N ORF yielding
a novel recombinant human-bovine chimeric PIV cDNA clone. Replacement of the
HPIV3 N ORF of HPIV3 with that of BPIV3 Ka or SF results in a protein with

5      approximately 70 amino acid differences (depending on the strain involved) from that of
HPIV3 N.  N is one of the more conserved proteins, and substitution of other proteins
such as P, singly or in combination, would result in many more amino acid differences.
The involvement of multiple genes and genome segments each conferring multiple amino
acid or nucleotide differences provides a broad basis for attenuation which is highly

10     stable to reversion.

       This mode of attenuation contrasts sharply to current HPIV vaccine
candidates that are attenuated by one or more point mutations, where reversion of an
individual mutation may yield a significant or complete reacquisition of virulence. In
addition, several known attenuating point mutations in HPIV typically yield a temperature

15     sensitive phenotype. One problem with attenuation associated with temperature
sensitivity is that the virus can be overly restricted for replication in the lower respiratory
tract while being under attenuated in the upper respiratory tract. This is because there is a
temperature gradient within the respiratory tract, with temperature being higher (and more
restrictive) in the lower respiratory tract and lower (less restrictive) in the upper

20     respiratory tract.  The ability of an attenuated virus to replicate in the upper respiratory
tract can result in complications including congestion, rhinitis, fever and otitis media,
whereas overattenuation in the lower respiratory tract can reduce immunogenicity. Thus,
attenuation achieved solely by temperature sensitive mutations may not be ideal. In
contrast, host range mutations present in human-bovine chimeric PIV of the invention

25     will not in most cases confer temperature sensitivity. Therefore, the novel method of PIV
attenuation provided by the invention will be more stable genetically and phenotypically
and less likely to be associated with residual virulence in the upper respiratory tract
compared to other known PIV vaccine candidates.

       Surprisingly, both the Ka and SF HPIV3/BPIV3 chimeric recombinants

30     involving the N ORF replacement were viable. Since the N protein of Ka or SF strain
BPIV3 differs in 70 of 515 amino acid residues, respectively, from that of the JS strain of
HPIV3. It was therefore unexpected that a bovine N protein with this level of amino acid
sequence divergence could efficiently interact with the HPIV3 RNA, or with other HPIV3
proteins that constitute the functional replicase/transcriptase. Equally surprising was the

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finding that the Ka and SF chimeric viruses replicated as efficiently in cell culture as either HPIV3 or BPIV3 parent indicating that the chimeric recombinants did not exhibit gene incompatibilities that restricted replication *in vitro*. This property of efficient replication *in vitro* is important since it permits efficient manufacture of this biological.

5          Also surprising is the observation, based on the studies hereinbelow, that the Ka and the SF HPIV3/BPIV3 chimeric recombinants (termed cKa and cSF), bearing only one bovine gene, are nearly equivalent to their BPIV3 parents in the degree of host range restriction in the respiratory tract of the rhesus monkey. In particular, the cKa and cSF viruses exhibited approximately a 60-fold or 30-fold reduction, respectively, in

10         replication in the upper respiratory tract of rhesus monkeys compared to replication of HPIV3. Based on this finding, it is possible that other BPIV3 genes will also confer desired levels of host range restriction within human-bovine chimeric PIV of the invention. Thus, according to the methods herein, a list of attenuating determinants will be readily identified in heterologous genes and genome segments of both HPIV and BPIV

15         that will confer, in appropriate combination, an optimal level of host range restriction and immunogenicity on human-bovine chimeric PIV selected for vaccine use. In preferred vaccine recombinants, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model for PIV replication in humans, e.g., hamsters or rhesus monkeys, may be reduced by at least about two-fold, more often about

20         5-fold, 10-fold, or 20-fold, and preferably 50-100-fold and up to 1,000-fold or greater overall (e.g., as measured between 3-8 days following infection) compared to growth of the corresponding wild-type or mutant parental PIV strain.

Confirming the unexpected nature and advantages provided by the human-bovine chimeric PIV of the invention, both the cKa and cSF induced a high level of

25         protection against HPIV3 challenge in the respiratory tract of rhesus monkeys, despite the exceptional degree of restriction of replication exhibited by these viruses in this model for human PIV infection and protection. In particular, previous infection with either chimeric virus induced a high level of resistance to replication of the rJS challenge virus in both the upper and lower respiratory tract. Infection of monkeys with cKa elicited a high degree

30         of protection as indicated by an approximate 300-fold reduction of replication of wild type HPIV3 (rJS) in the upper respiratory tract, and an approximate 1000-fold reduction in the lower tract compared to uninoculated control monkeys. Monkeys infected with cSF manifested a 2000-fold reduction of replication of rJS in the upper respiratory tract, and a 1000-fold reduction in the lower tract compared to uninoculated control monkeys.

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The levels of protection elicited by cKa or cSF were comparable to those seen in
monkeys previously infected with either the bovine or the human PIV parent.  Thus,
infection with human-bovine chimeric PIV of the invention provides a high level of
protection in the upper and lower respiratory tract of monkeys, and both chimeric viruses

5    represent promising vaccine candidates.  In other preferred vaccine recombinants, the
immunogenic activity of human-bovine chimeric PIV will be balanced against the level of
attenuation to achieve useful vaccine candidates, and will typically be marked by a
reduction of replication of challenge virus, e.g., rJS in the lower and/or upper respiratory
tract by about 50-100-fold, 100-500-fold, preferably about 500-2,000-fold and up to

10   3,000-fold or greater overall (e.g., as measured between 3-8 days post-challenge).  Thus,
the recombinant vaccine viruses of the invention maintain immunogenicity while
exhibiting concomitant reductions in replication and growth.  This surprising assemblage
of phenotypic traits is highly desired for vaccine development.

        The observation that the N gene from two independent strains of BPIV3

15   confers an attenuation phenotype on HPIV3 for the rhesus monkey indicates that this is
likely a property shared by N genes of other BPIV strains.  Accordingly, within the
methods of the invention any BPIV gene or genome segment, singly or in combination
with one or more other BPIV gene(s) or genome segment(s), can be combined with HPIV
sequences to produce an attenuated HPIV3/BPIV3 chimeric recombinant virus suitable

20   for use as a vaccine virus.  In preferred embodiments, all HPIVs, including HPIV1,
HPIV2, HPIV3 and variant strains thereof, are useful recipients for attenuating BPIV
gene(s) and/or genome segment(s).  In general, the HPIV genes selected for inclusion in a
HPIV3/BPIV3 chimeric virus will include one or more of the protective antigens, such as
the HN or F glycoproteins.

25           Alternative human-bovine chimeric PIV of the invention will contain
protective antigenic determinants of HPIV1 or HPIV2.  This may be achieved, for
example, by expression of an HN and/or F gene of HPIV1 or HPIV2 as an extra gene(s)
in an attenuated HPIV3/BPIV3 chimeric recombinant.  Alternatively, it is possible to use
a HPIV3/HPIV1 or a HPIV3/HPIV2 antigenic chimeric virus, in which the HPIV1 or

30   HPIV2 HN and/or F genes replace their PIV3 counterpart(s) (Skiadopoulos et al., 1999a,
*supra*; Tao et al., 1999, *supra*; and U.S. Patent Application Serial No. 09/083,793, filed
May 22, 1998; each incorporated herein by reference), as a recipient or background virus
for one or more heterologous, attenuating bovine gene(s) or genome segment(s), for
example a Ka or SF N gene or genome segment.  Such antigenic chimeric viruses will be

attenuated by the bovine N gene, but will induce immunity to the HPIV1 or HPIV2 virus. In this context, a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in

5    L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp*45L (Skiadopoulos et al., 1998, *supra*; Tao et al., 1998, *supra*; Tao et al., 1999, *supra*). rPIV3-1.cp45L was attenuated in hamsters and induced a high level of resistance to challenge with PIV1. A recombinant chimeric virus, designated rPIV3-1*cp*45, has also been produced that contains 12 of the 15 *cp*45 mutations, i.e., excluding the mutations in

10   HN and F, and is highly attenuated in the upper and lower respiratory tract of hamsters (Skiadopoulos et al., 1999a, *supra*).

Still other HPIV/BPIV chimeric recombinants will incorporate two or more BPIV genes or genome segments, in any combination, up to and including all of the BPIV genome other than selected genes or antigenic determinants selected from HN or F

15   gene(s) and genome segment(s), which could come from a human HPIV1, HPIV2, or HPIV3 virus. Yet additional embodiments of the invention are directed to human-bovine chimeric PIV incorporating attenuating genes from other animal PIVs, such as murine PIV1, the canine SV5 PIV2 virus, or another avian or mammalian PIV in combination with a HPIV backbone, alternatively including a chimeric HPIV backbone, from HPIV1,

20   HPIV2, and/or HPIV3.

In other detailed aspects of the invention, human-bovine chimeric PIV are employed as vectors for protective antigens of heterologous pathogens, including other PIVs and non-PIV viruses and non-viral pathogens. Within these aspects, the bovine-human chimeric genome or antigenome comprises a partial or complete PIV "vector

25   genome or antigenome" combined with one or more heterologous genes or genome segments encoding one or more antigenic determinants of one or more heterologous pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference). The heterologous pathogen in this context may be a heterologous PIV and the heterologous

30   gene(s) or genome segment(s) can be selected to encodes one or more PIV N, P, C, D, V, M, F, SH (where applicable), HN and/or L protein(s), as well as protein domains, fragments, and immunogenic regions or epitopes. PIV vector vaccines thus constructed may elicit a polyspecific immune response and may be administered simultaneously or in a coordinate adminstration protocol with other vaccine agents.

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In exemplary embodiments of the invention, human-bovine chimeric PIV
may comprise a vector genome or antigenome that is a partial or complete HPIV genome
or antigenome, which is combined with or is modified to incorporate one or more
heterologous genes or genome segments encoding antigenic determinant(s) of one or

5      more heterologous PIV(s), including heterologous HPIVs selected from HPIV1, HPIV2,
or HPIV3. In more detailed aspects, the vector genome or antigenome is a partial or
complete HPIV3 genome or antigenome and the heterologous gene(s) or genome
segment(s) encoding the antigenic determinant(s) is/are of one or more heterologous
HPIV(s). Typically, the chimeric genome or antigenome incorporates one or more

10     gene(s) or genome segment(s) of a BPIV that specifies attenuation.

In exemplary aspects of the invention, the bovine-human chimeric PIV
incorporates one or more HPIV1 or HPIV2 genes or genome segments that encode(s) one
or more HN and/or F glycoproteins or antigenic domains, fragments or epitopes thereof
within a partial or complete HPIV3 vector genome or antigenome. In more detailed

15     aspects, both HPIV1 genes encoding HN and F glycoproteins are substituted for
counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or
antigenome. Such recombinant constructs can be used to produce vaccine virus directly,
or can be further modified by addition or incorporation of one or more genes or gene
segments encoding one or more antigenic determinants. Such constructs for the

20     production of vaccine viruses typically incorporate one or more heterologous gene(s) or
genome segment(s) of a BPIV that specifies attenuation, for example an open reading
frame (ORF) encoding an attenuating BPIV protein, such as N. Certain human-bovine
chimeric PIV of the invention may be employed as vectors for generating specific
vaccines to HPIV2, for example wherein a transcription unit comprising an open reading

25     frame (ORF) of an HPIV2 HN gene is added to or incorporated within a chimeric HPIV3-
1 vector genome or antigenome and the chimeric construct is attenuated by incorporation
of a BPIV gene or genome segment.

Within related aspects of the invention, the vector genome or antigenome
is a partial or complete BPIV genome or antigenome, and the heterologous genes or

30     genome segments encoding the antigenic determinant(s) is/are of one or more HPIV(s).
Typically, the determinant(s) is/are selected from HPIV1, HPIV2 or HPIV3 HN and F
glycoproteins, but antigenic domains, fragments and epitopes of these and other antigenic
proteins are also useful. In certain embodiments, one or more genes or genome segments
encoding one or more antigenic determinant(s) of HPIV2 is/are added to or substituted

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within the partial or complete BPIV vector genome or antigenome. Alternatively, a plurality of heterologous genes or genome segments encoding antigenic determinants of multiple HPIVs may be added to or incorporated within the partial or complete BPIV vector genome or antigenome.

5          In yet additional aspects of the invention, human-bovine chimeric PIV are provided as vectors for a range of non-PIV pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference). The vector genome or antigenome for use within these aspects of the invention may comprise a partial or complete BPIV or HPIV genome or

10        antigenome, and the heterologous pathogen may be selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses.

15        For example, a HPIV or BPIV vector genome or antigenome for constructing bovine-human chimeric PIV of the invention may incorporate heterologous antigenic determinant(s) selected from the measles virus HA and F proteins, or antigenic domains, fragments and epitopes thereof. In exemplary embodiments, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to or

20        incorporated within a BPIV or HPIV3 vector genome or antigenome.

Alternatively bovine-human chimeric PIV of the invention may used as vectors to incorporate heterologous antigenic determinant(s) from respiratory syncytial virus (RSV), for example by incorporating one or more genes or genome segments that encode(s) RSV F and/or G glycoprotein or immunogenic domain(s) or epitope(s) thereof.

25        In this context, the cloning of RSV cDNA and other disclosure is provided in U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed

30        May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to International Publication No. WO 98/02530); U.S. Patent Application Serial No. 09/291,894, filed on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999; Collins, et al., 1995, *supra*; Bukreyev, et al., J. Virol. 70:6634-6641, 1996; Juhasz et al., 1997, *supra*; Durbin et al., 1997a, *supra*; He et

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al., 1997, *supra*; Baron et al., 1997, *supra*; Whitehead et al., 1998a, *supra*; Whitehead et al., 1998b, *supra*; Jin et al., 1998, *supra*; and Whitehead et al., 1999, *supra*; and Bukreyev et al., Proc. Natl. Acad. Sci. USA 96:2367-2372, 1999, each incorporated herein by reference in its entirety for all purposes).

5             According to this aspect of the invention, human-bovine chimeric PIV are provided which incorporate at least one antigenic determinant from a heterologous PIV or non-PIV pathogen. For example, one or more individual gene(s) or genome segment(s) of HPIV3 may be replaced with counterpart gene(s) or genome segment(s) from human RSV, or an RSV gene or genome segment can be inserted or added as an supernumerary

10       gene. Alternatively, a selected, heterologous genome segment, e.g. encoding a cytoplasmic tail, transmembrane domain or ectodomain of an RSV glycoprotein, is substituted for a counterpart genome segment in, e.g., the same gene in HPIV3 or within a different gene in HPIV3, or added within a non-coding sequence of the HPIV3 genome or antigenome to yield a chimeric PIV-RSV glycoprotein. In one embodiment, a genome

15       segment from an F gene of human RSV is substituted for a counterpart HPIV3 genome segment to yield constructs encoding chimeric proteins, e.g. fusion proteins having a cytoplasmic tail and/or transmembrane domain of PIV fused to an ectodomain of RSV to yield a novel attenuated virus, and/or a multivalent vaccine immunogenic against both PIV and RSV.

20             As noted above, it is often desirable to adjust the attenuation phenotype in human-bovine chimeric PIV of the invention by introducing additional mutations that increase or decrease attenuation or otherwise alter the phenotype of the chimeric virus. Detailed descriptions of the materials and methods for producing recombinant PIV from cDNA, and for making and testing the full range of mutations and nucleotide

25       modifications set forth herein as supplemental aspects of the present invention, provided in, e.g., Durbin et al., 1997a, *supra*; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997; each incorporated herein by

30       reference. In particular, these documents describe methods and procedures for mutagenizing, isolating and characterizing PIV to obtain attenuated mutant strains (e.g., temperature sensitive (ts), cold passaged (cp) cold-adapted (ca), small plaque (sp) and host-range restricted (hr) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype. In conjunction with these methods, the foregoing

documents detail procedures for determining replication, immunogenicity, genetic

stability and protective efficacy of biologically derived and recombinantly produced

attenuated human PIV in accepted model systems, including murine and non-human

primate model systems. In addition, these documents describe general methods for

5      developing and testing immunogenic compositions, including monovalent and bivalent

vaccines, for prophylaxis and treatment of PIV infection. Methods for producing

infectious recombinant PIV by construction and expression of cDNA encoding a PIV

genome or antigenome coexpressed with essential PIV proteins are also described in the

above-incorporated documents, which include description of the following exemplary

10     plasmids that may be employed to produce infectious PIV viral clones: p3/7(131) (ATCC

97990); p3/7(131)2G (ATCC 97889); and p218(131) (ATCC 97991); each deposited

under the terms of the Budapest Treaty with the American Type Culture Collection

(ATCC) of 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and

granted the above identified accession numbers.

15                   Also disclosed in the above-incorporated references are methods for

constructing and evaluating infectious recombinant PIV that are modified to incorporate

phenotype-specific mutations identified in biologically-derived PIV mutants, e.g., cold

passaged (cp), cold adapted (ca), host range restricted (hr), small plaque (sp), and/or

temperature sensitive (ts) mutants, for example the JS HPIV3 cp 45 mutant strain. Other

20     mutations may be attenuating without an auxiliary marker phenotype. Mutations

identified in these mutants can be readily adopted in human-bovine chimeric PIV. In

exemplary embodiments, one or more attenuating mutations occur in the polymerase L

protein, e.g., at a position corresponding to $Tyr_{942}$, $Leu_{992}$, or $Thr_{1558}$ of JS cp45.

Preferably, these mutations are incorporated in human-bovine chimeric PIV of the

25     invention by an identical, or conservative, amino acid substitution as identified in the

biological mutant. Thus, PIV recombinants may incorporate a mutation wherein $Tyr_{942}$ is

replaced by His, $Leu_{992}$ is replaced by Phe, and/or $Thr_{1558}$ is replaced by Ile.

Substitutions that are conservative to these replacement amino acids are also useful to

achieve a desired mutant phenotype

30                   Other exemplary mutations adopted from a biologically derived PIV

mutant include one or more mutations in the N protein, including specific mutations at a

position corresponding to residues $Val_{96}$ or $Ser_{389}$ of JS cp45. In more detailed aspects,

these mutations are represented as $Val_{96}$ to Ala or $Ser_{389}$ to Ala or substitutions that are

conservative thereto. Also useful within recombinant PIV of the invention are amino acid

substitution in the C protein, e.g., a mutation at a position corresponding to $Ile_{96}$ of JS *cp*45, preferably represented by an identical or conservative substitution of $Ile_{96}$ to Thr. Further exemplary mutations adopted from biologically derived PIV mutants include one mutation in the M gene such as $Pro_{199}$ in JS *cp*45, or more mutations in the F protein,

5      including mutations adopted from JS *cp*45 at a position corresponding to residues Ile*420* or $Ala_{450}$ of JS *cp*45, preferably represented by acid substitutions $Ile_{420}$ to Val or $Ala_{450}$ to Thr or substitutions conservative thereto. Other human-bovine chimeric PIV within the invention adopt one or more amino acid substitutions in the HN protein, as exemplified hereinbelow by a recombinant PIV adopting a mutation at a position corresponding to

10     residue $Val_{384}$ of JS, preferably represented by the substitution $Val_{384}$ to Ala.

Yet additional examples within this aspect of the invention include human-bovine chimeric PIV which incorporate one or more mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant

15     virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS cp45. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS cp45. In more detailed aspects human-bovine chimeric PIV incorporate a T to C change at nucleotide 23, a C to T change at

20     nucleotide 24, a G to T change at nucleotide 28, and/or a T to A change at nucleotide 45. Additional mutations in extragenic sequences are exemplified by a A to T change in N gene start sequence at a position corresponding to nucleotide 62 of JS.

These foregoing exemplary mutations which can be engineered in a human-bovine chimeric PIV of the invention have been successfully engineered and

25     recovered in recombinant PIV, as represented by the recombinant PIV clones designated rcp45, rcp45 L, rcp45 F, rcp45 M, rcp45 HN, rcp45 C, rcp45 F, rcp45 3'N, 3'NL, and rcp45 3'NCMFHN (Durbin et al., 1997a, *supra*; Skiadopolos et al., 1998, *supra*; Skiadopolos et al., J. Virol. 73:1374-1381, 1999b; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed

30     May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference). In addition, the above-incorporated references describe construction of chimeric PIV recombinants, e.g., having the HN and F genes of HPIV1 substituted into a partial HPIV3 background genome or antigenome, which is further modified to bear one

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or more of the attenuating mutations identified in HPIV3 JS cp45. One such chimeric recombinant incorporates all of the attenuating mutations identified in the L gene of cp45. It has since been shown that all of the cp45 mutations outside of the heterologous (HPIV1) HN and F genes can be incorporated in a HPIV3-1 recombinant to yield an

5      attenuated, chimeric vaccine candidate.

From JS cp45 and other biologically derived PIV mutants, a large "menu" of attenuating mutations is provided, each of which can be combined with any other mutation(s) for adjusting the level of attenuation, immunogenicity and genetic stability in a recombinant PIV bearing C, D, and/or V deletion or knock out mutation(s). In this

10     context, many recombinant PIVs of the invention will include one or more, and preferably two or more, mutations from biologically derived PIV mutants, e.g., any one or combination of mutations identified in JS cp45. Preferred PIV recombinants within the invention will incorporate a plurality and up to a full complement of the mutations present in JS cp45 or other biologically derived mutant PIV strains. Preferably, these mutations

15     are stabilized against reversion in human-bovine chimeric PIV by multiple nucleotide substitutions in a codon specifying each mutation.

Additional mutations that may be incorporated in human-bovine chimeric PIV of the invention are mutations, e.g., attenuating mutations, identified in heterologous PIV or more distantly related nonsegmented negative stranded RNA viruses. In

20     particular, attenuating and other desired mutations identified in one negative stranded RNA virus may be "transferred", e.g., introduced by mutagenesis in a corresponding position within the genome or antigenome of the human-bovine chimeric PIV. Briefly, desired mutations in one heterologous negative stranded RNA virus are transferred to the PIV recipient (e.g., bovine or human PIV, respectively). This involves mapping the

25     mutation in the heterologous virus, thus identifying by sequence alignment the corresponding site in the recipient RSV, and mutating the native sequence in the PIV recipient to the mutant genotype (either by an identical or conservative mutation), as described in PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by

30     reference). As this disclosure teaches, it is preferable to modify the recipient genome or antigenome to encode an alteration at the subject site of mutation that corresponds conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a similar substitution should be engineered at the

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corresponding residue(s) in the recombinant virus. Preferably the substitution will involve an identical or conservative amino acid to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant

5    protein (e.g., by using any other amino acid to disrupt or impair the function of the wild-type residue).

Negative stranded RNA viruses from which exemplary mutations are identified and transferred into human-bovine chimeric PIV of the invention include other PIVs (e.g., HPIV1, HPIV2, HPIV3, HPIV4A, HPIV4B and BPIV3), RSV, Sendai virus

10   (SeV), Newcastle disease virus (NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV), rabies virus (RaV) and vesicular stomatitis virus (VSV), among others.

A variety of exemplary mutations for use within the invention are disclosed in the above-incorporated reference, including but not limited to an amino acid

15   substitution of phenylalanine at position 521 of the RSV L protein corresponding to and therefore transferable to a substitution of phenylalanine (or a conservatively related amino acid) at position 456 of the HPIV3 L protein. In the case of mutations marked by deletions or insertions, these can be introduced as corresponding deletions or insertions into the recombinant virus, either within the background genome or antigenome or within

20   the heterologous gene or genome segment incorporated therein. However the particular size and amino acid sequence of the deleted or inserted protein fragment can vary.

Yet additional human-bovine PIV vaccine candidates within the invention can be achieved by modifying the chimeric PIV genome or antigenome to encode an analogous mutation to an attenuating mutation identified in Sendai virus (SeV). In one

25   example, the attenuating mutation comprises an amino acid substitution of phenylalanine at position 170 of the C protein of SeV. The PIV genome or antigenome is modified to encode an alteration of a conserved residue that corresponds conservatively to the alteration marking the attenuating mutation in the heterologous, SeV mutant. In one embodiment, the mutation is incorporated within a recombinant HPIV3 protein and

30   comprises an amino acid substitution of phenylalanine at position 164 of the C protein of HPIV3.

Various target proteins are amenable to introduction of attenuating mutations from one negative stranded RNA virus at a corresponding site within chimeric human-bovine PIV of the invention. Throughout the order Mononegavirales, five target

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proteins are strictly conserved and show moderate to high degrees of sequence identity

for specific regions or domains.  In particular, all known members of the order share a

homologous constellation of five proteins: a nucleocapsid protein (N), a nucleocapsid

phosphoprotein (P), a nonglycosylated matrix (M) protein, at least one surface

5      glycoprotein (HN, F, H, or G) and a large polymerase (L) protein.  These proteins all

represent useful targets for incorporating attenuating mutations by altering one or more

conserved residues in a protein of the recombinant virus at a site corresponding to the site

of an attenuating mutation identified in the heterologous, mutant virus.

In this context, the methods for transferring heterologous mutations into

10     chimeric human-bovine PIV of the invention are based on identification of an attenuating

mutation in a first negative stranded RNA virus.  The mutation, identified in terms of

mutant versus wild-type sequence at the subject amino acid position(s) marking the site of

the mutation, provides an index for sequence comparison against a homologous protein in

the chimeric virus (either in the background genome or antigenome or in the heterologous

15     gene or gene segment added or substituted therein) that is the target for recombinant

attenuation.  The attenuating mutation may be previously known or may be identified by

mutagenic and reverse genetics techniques applied to generate and characterize

biologically-derived mutant virus.  Alternatively, attenuating mutations of interest may be

generated and characterized *de novo*, e.g., by site directed mutagenesis and conventional

20     screening methods.

Each attenuating mutation identified in a negative stranded RNA virus

provides an index for sequence comparison against a homologous protein in one or more

heterologous negative stranded virus(es).  In this context, existing sequence alignments

may be analyzed, or conventional sequence alignment methods may be employed to yield

25     sequence comparisons for analysis, to identify corresponding protein regions and amino

acid positions between the protein bearing the attenuating mutation and a homologous

protein of a different virus that is the target recombinant virus for attenuation.  Where one

or more residues marking the attenuating mutation have been altered from a "wild-type"

identity that is conserved at the corresponding amino acid position(s) in the target human-

30     bovine chimeric virus protein, the genome or antigenome of the target virus is

recombinantly modified to encode an amino acid deletion, substitution, or insertion to

alter the conserved residue(s) in the target virus protein and thereby confer an analogous,

attenuated phenotype on the recombinant virus.

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Within this rational design method for constructing attenuated recombinant negative stranded viruses, the wild-type identity of residue(s) at amino acid positions marking an attenuating mutation in one negative stranded RNA virus may be conserved strictly, or by conservative substitution, at the corresponding amino acid position(s) in the

5    target, human-bovine chimeric virus protein. Thus, the corresponding residue(s) in the target virus protein may be identical, or may be conservatively related in terms of amino acid side-group structure and function, to the wild-type residue(s) found to be altered by the attenuating mutation in the heterologous, mutant virus. In either case, analogous attenuation in the recombinant virus may be achieved according to the methods of the

10   invention by modifying the recombinant genome or antigenome of the target virus to encode the amino acid deletion, substitution, or insertion to alter the conserved residue(s).

In this context, it is preferable to modify the genome or antigenome to encode an alteration of the conserved residue(s) that corresponds conservatively to the alteration marking the attenuating mutation in the heterologous, mutant virus. For

15   example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a substitution should be engineered at the corresponding residue(s) in the recombinant virus. Preferably the substitution will be identical or conservative to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at

20   the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (e.g., by using any other amino acid to disrupt or impair the identity and function of the wild-type residue). In the case of mutations marked by deletions or insertions, these can transferred as corresponding deletions or insertions into the recombinant virus, however the particular size and amino acid sequence of the deleted or inserted protein

25   fragment can vary.

Within alternative aspects of the invention, mutations thus transferred from heterologous mutant negative stranded viruses may confer a variety of phenotypes within human-bovine chimeric PIV of the invention, in addition to or associated with the desired, an attenuated phenotype. Thus, exemplary mutations incorporated within

30   recombinant proteins of the virus may confer temperature sensitive (*ts*), cold-adapted (*ca*), small plaque (*sp*), or host range restricted (*hr*) phenotypes, or a change in growth or immunogenicity, in addition to or associated with the attenuated phenotype.

Attenuating mutations in biologically derived PIV and other nonsegmented negative stranded RNA viruses for incorporation within human-bovine chimeric PIV may

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occur naturally or may be introduced into wild-type PIV strains by well known

mutagenesis procedures. For example, incompletely attenuated parental PIV strains can

be produced by chemical mutagenesis during virus growth in cell cultures to which a

chemical mutagen has been added, by selection of virus that has been subjected to

5      passage at suboptimal temperatures in order to introduce growth restriction mutations, or

by selection of a mutagenized virus that produces small plaques (sp) in cell culture, as

described in the above incorporated references.

By "biologically derived PIV" is meant any PIV not produced by

recombinant means. Thus, biologically derived PIV include all naturally occurring PIV,

10     including, e.g., naturally occurring PIV having a wild-type genomic sequence and PIV

having allelic or mutant genomic variations from a reference wild-type RSV sequence,

e.g., PIV having a mutation specifying an attenuated phenotype. Likewise, biologically

derived PIV include PIV mutants derived from a parental PIV by, *inter alia*, artificial

mutagenesis and selection procedures.

15     As noted above, production of a sufficiently attenuated biologically

derived PIV mutant can be accomplished by several known methods. One such

procedure involves subjecting a partially attenuated virus to passage in cell culture at

progressively lower, attenuating temperatures. For example, partially attenuated mutants

are produced by passage in cell cultures at suboptimal temperatures. Thus, a cp mutant or

20     other partially attenuated PIV strain is adapted to efficient growth at a lower temperature

by passage in culture. This selection of mutant PIV during cold-passage substantially

reduces any residual virulence in the derivative strains as compared to the partially

attenuated parent.

Alternatively, specific mutations can be introduced into biologically

25     derived PIV by subjecting a partially attenuated parent virus to chemical mutagenesis,

e.g., to introduce ts mutations or, in the case of viruses which are already ts, additional ts

mutations sufficient to confer increased attenuation and/or stability of the ts phenotype of

the attenuated derivative. Means for the introduction of ts mutations into PIV include

replication of the virus in the presence of a mutagen such as 5-fluorouridine according to

30     generally known procedures. Other chemical mutagens can also be used. Attenuation

can result from a ts mutation in almost any PIV gene, although a particularly amenable

target for this purpose has been found to be the polymerase (L) gene.

The level of temperature sensitivity of replication in exemplary attenuated

PIV for use within the invention is determined by comparing its replication at a

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permissive temperature with that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the shutoff temperature. In experimental animals and humans, both the replication and virulence of

5    PIV correlate with the mutant's shutoff temperature.

The JS cp45 HPIV3 mutant has been found to be relatively stable genetically, highly immunogenic, and satisfactorily attenuated. Nucleotide sequence analysis of this biologically derived and recombinant viruses incorporating various individual and combined mutations found therein, indicates that each level of increased

10    attenuation is associated with specific nucleotide and amino acid substitutions. The above-incorporated references also disclose how to routinely distinguish between silent incidental mutations and those responsible for phenotype differences by introducing the mutations, separately and in various combinations, into the genome or antigenome of infectious PIV clones. This process coupled with evaluation of phenotype characteristics

15    of parental and derivative virus identifies mutations responsible for such desired characteristics as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc.

Mutations thus identified are compiled into a "menu" and are then introduced as desired, singly or in combination, to adjust a human-bovine chimeric PIV to

20    an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc., as desired. In accordance with the foregoing description, the ability to produce infectious PIV from cDNA permits introduction of specific engineered changes within human-bovine chimeric PIV. In particular, infectious, recombinant PIVs are employed for identification of specific mutation(s) in biologically

25    derived, attenuated PIV strains, for example mutations which specify ts, ca, att and other phenotypes. Desired mutations are thus identified and introduced into recombinant, human-bovine chimeric PIV vaccine strains. The capability of producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, whereafter the phenotypes of

30    rescued recombinant viruses containing the introduced mutations can be readily determined.

By identifying and incorporating specific, biologically derived mutations associated with desired phenotypes, e.g., a cp or ts phenotype, into infectious PIV clones, the invention provides for other, site-specific modifications at, or within close proximity

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to, the identified mutation. Whereas most attenuating mutations produced in biologically derived PIV are single nucleotide changes, other "site specific" mutations can also be incorporated by recombinant techniques into biologically derived or recombinant PIV. As used herein, site-specific mutations include insertions, substitutions, deletions or

5    rearrangements of from 1 to 3, up to about 5-15 or more altered nucleotides (e.g., altered from a wild-type PIV sequence, from a sequence of a selected mutant PIV strain, or from a parent recombinant PIV clone subjected to mutagenesis). Such site-specific mutations may be incorporated at, or within the region of, a selected, biologically derived point mutation. Alternatively, the mutations can be introduced in various other contexts within

10   a PIV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc. Site-specific PIV mutants typically retain a desired attenuating phenotype, but may additionally exhibit altered phenotypic characteristics unrelated to attenuation, e.g., enhanced or broadened immunogenicity, and/or improved growth. Further examples of

15   desired, site-specific mutants include recombinant PIV designed to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid changes in a parent mutant or recombinant PIV clone, yielding a biologically derived or recombinant PIV having genetic resistance to reversion

20   from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream or downstream, e. g., from 1 to 3, 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, e.g., to construct or ablate an existing cis-acting regulatory element.

25          In addition to single and multiple point mutations and site-specific mutations, changes to the human-bovine chimeric PIV disclosed herein include deletions, insertions, substitutions or rearrangements of one or more gene(s) or genome segment(s). Particularly useful are deletions involving one or more gene(s) or genome segment(s), which deletions have been shown to yield additional desired phenotypic effects for

30   adjusting the characteristics of human-bovine chimeric PIV within the invention. Thus, U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999) describes methods and compositions whereby expression of one or more HPIV genes, exemplified by the C, D, and/or V ORFs, is reduced or ablated by modifying the PIV genome or antigenome to incorporate a mutation that alters the coding assignment of an

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initiation codon or mutation(s) that introduce one or one or more stop codon(s). Alternatively, one or more of the C, D, and/or V ORFs can be deleted in whole or in part to render the corresponding protein(s) partially or entirely non-functional or to disrupt protein expression altogether. Recombinant PIV having such mutations in C, D, and/or

5    V, or other non-essential gene(s), possess highly desirable phenotypic characteristics for vaccine development. For example, these modifications may specify one or more desired phenotypic changes including (i) altered growth properties in cell culture, (ii) attenuation in the upper and/or lower respiratory tract of mammals, (iii) a change in viral plaque size, (iv) a change in cytopathic effect, and (v) a change in immunogenicity. One such

10   exemplary "knock out" mutant lacking C ORF expression, designated rC-KO, was able to induce a protective immune response against wild type HPIV3 challenge in a non-human primate model despite its beneficial attenuation phenotype.

Thus, in more detailed aspects of the instant invention, human-bovine chimeric PIV incorporate deletion or knock out mutations in a C, D, and/or V ORF(s)

15   which alters or ablates expression of the selected gene(s) or genome segment(s). This can be achieved, e.g., by introducing a frame shift mutation or termination codon within a selected coding sequence, altering translational start sites, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, changing GS and/or GE transcription signals to alter phenotype, or modifying an RNA editing site (e.g.,

20   growth, temperature restrictions on transcription, etc.). In more detailed aspects of the invention, human-bovine chimeric PIVs are provided in which expression of one or more gene(s), e.g., a C, D, and/or V ORF(s), is ablated at the translational or transcriptional level without deletion of the gene or of a segment thereof, by, e.g., introducing multiple translational termination codons into a translational open reading frame (ORF), altering

25   an initiation codon, or modifying an editing site. These forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, these methods provide yet additional, novel types of attenuating mutations which ablate expression of a viral gene that is not one of the major viral protective antigens. In this context, knock-out virus phenotypes produced without deletion of a gene or genome

30   segment can be alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore synthesis of a target protein. Several other gene knock-outs for the C, D, and/or V ORF(s) deletion and knock out mutants can be made using alternate designs and methods that are well known in the art (as described, for example, in (Kretschmer et al., Virology 216:309-316, 1996; Radecke

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et al., <u>Virology</u> <u>217</u>:418-421, 1996; and Kato et al., 1987a, *supra*; and Schneider et al., 1997, *supra*; each incorporated herein by reference).

These and other nucleotide modifications in human-bovine chimeric PIV may alter small numbers of bases (e.g., from 15-30 bases, up to 35-50 bases or more),
5    large blocks of nucleotides (e.g., 50-100, 100-300, 300-500, 500-1,000 bases), or nearly complete or complete genes (e.g., 1,000-1,500 nucleotides, 1,500-2,500 nucleotides, 2,500-5,000, nucleotides, 5,000-6,500 nucleotides or more) in the donor or recipient genome or antigenome, depending upon the nature of the change (i.e., a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small
10   genome segment, whereas large block(s) of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged.

In related aspects, the invention provides for supplementation of mutations adopted into a recombinant PIV clone from biologically derived PIV, e.g., cp and ts mutations, with additional types of mutations involving the same or different genes in a
15   further modified PIV clone.  Each of the PIV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a human-bovine chimeric PIV exhibiting novel vaccine characteristics.  Thus, in addition to or in combination with attenuating mutations adopted from biologically derived PIV mutants,
20   the present invention also provides a range of additional methods for attenuating or otherwise modifying the phenotype of human-bovine chimeric PIV based on recombinant engineering of infectious PIV clones.  A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a chimeric PIV genome or antigenome
25   for incorporation into infectious clones.  More specifically, to achieve desired structural and phenotypic changes in recombinant PIV, the invention allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected nucleotide or plurality of nucleotides from a parent genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole gene(s) or genome segment(s), within a
30   human-bovine chimeric PIV clone.

Thus provided are modifications in the human-bovine chimeric PIV which simply alter or ablate expression of a selected gene, e.g., by introducing a termination codon within a selected PIV coding sequence or altering its translational start site or RNA editing site, changing the position of a PIV gene relative to an operably linked promoter,

introducing an upstream start codon to alter rates of expression, modifying (e.g., by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE transcription signals to alter phenotype (e.g., growth, temperature restrictions on transcription, etc.), and various other deletions,

5    substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected gene(s), or translation of selected RNA(s). In this context, any PIV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant PIV to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. As for

10   coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, e.g., by the use of minireplicons and recombinant PIV.

In addition, a variety of other genetic alterations can be produced in a PIV genome or antigenome for incorporation into human-bovine chimeric PIV, alone or

15   together with one or more attenuating mutations adopted from a biologically derived mutant PIV, e.g., to adjust growth, attenuation, immunogenicity, genetic stability or provide other advantageous structural and/or phenotypic effects. These additional types of mutations are also disclosed in the foregoing incorporated references and can be readily engineered into human-bovine chimeric PIV of the invention.

20   In addition to these changes, the order of genes in a human-bovine chimeric PIV can be changed, a PIV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic

25   regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Other mutations for incorporation into human-bovine chimeric PIV of the invention include mutations directed toward cis-acting signals, which can be identified, e.g., by mutational analysis of PIV minigenomes. For example, insertional and deletional

30   analysis of the leader and trailer and flanking sequences identifies viral promoters and transcription signals and provides a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also has identified many mutations which affect RNA replication or transcription.

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Any of these mutations can be inserted into a human-bovine chimeric PIV antigenome or genome as described herein. Evaluation and manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by the use of PIV minigenomes as described in the above-incorporated references.

5        Additional mutations within the human-bovine chimeric PIV involve replacement of the 3' end of genome with its counterpart from antigenome, which is associated with changes in RNA replication and transcription. In one exemplary embodiment, the level of expression of specific PIV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which
10     have been made synthetically and designed to be consistent, with efficient translation. In this context, it has been shown that codon usage can be a major factor in the level of translation of mammalian viral proteins (Hans et al., Current Biol. 6:315-324, 1996, incorporated herein by reference). Optimization by recombinant methods of the codon usage of the mRNAs encoding the HN and F proteins of PIV, which are the major
15     protective antigens, will provide improved expression for these genes.

In another exemplary embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position) of a selected PIV gene is modified, alone or in combination with introduction of an upstream start codon, to modulate PIV gene expression by specifying up- or down-regulation of translation
20     (Kozak et al., J. Mol. Biol. 196:947-950, 1987). Alternatively, or in combination with other PIV modifications disclosed herein, gene expression of a human-bovine chimeric PIV can be modulated by altering a transcriptional GS or GE signal of any selected gene(s) of the virus. In alternative embodiments, levels of gene expression in the human-bovine chimeric PIV are modified at the level of transcription. In one aspect, the position
25     of a selected gene in the PIV gene map can be changed to a more promoter-proximal or promotor-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-fold or more compared to wild-type levels often attended by
30     a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel human-bovine chimeric PIV having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable properties such as increased antigen expression.

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Infectious human-bovine chimeric PIV clones of the invention can also be engineered according to the methods and compositions disclosed herein to enhance immunogenicity and induce a level of protection greater than that provided by infection with a wild-type PIV or a parent PIV. For example, an immunogenic epitope from a

5    heterologous PIV strain or type, or from a non-PIV source such as RSV, can be added to a recombinant clone by appropriate nucleotide changes in the polynucleotide sequence encoding the genome or antigenome. Alternatively, mutant PIV of the invention can be engineered to add or ablate (e.g., by amino acid insertion, substitution or deletion) immunogenic proteins, protein domains, or forms of specific proteins associated with

10   desirable or undesirable immunological reactions.

Within the methods of the invention, additional genes or genome segments may be inserted into or proximate to the human-bovine chimeric PIV genome or antigenome. These genes may be under common control with recipient genes, or may be under the control of an independent set of transcription signals. Genes of interest include

15   the PIV genes identified above, as well as non-PIV genes. Non-PIV genes of interest include those encoding cytokines (e.g., IL-2 through IL-18, especially IL-2, IL-6 and IL-12, IL-18, etc.). gamma-interferon, and proteins rich in T helper cell epitopes. These additional proteins can be expressed either as a separate protein, or as a supernumerary copy of an existing PIV proteins, such as HN or F. This provides the ability to modify

20   and improve the immune responses against PIV both quantitatively and qualitatively.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or genome segments within a human-bovine chimeric PIV yield highly stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of

25   resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (i.e., not essential for in vitro growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., 1997a, *supra*). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

30   In another aspect of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating human-bovine chimeric PIV-encoding cDNA) are provided for producing an isolated infectious PIV. Using these compositions and methods, infectious PIV are generated from a PIV genome or antigenome, a nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large (L) polymerase protein. In

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related aspects of the invention, compositions and methods are provided for introducing the aforementioned structural and phenotypic changes into a recombinant PIV to yield infectious, attenuated vaccine viruses.

Introduction of the foregoing defined mutations into an infectious, human-
5    bovine chimeric PIV clone can be achieved by a variety of well known methods. By "infectious clone" with regard to DNA is meant cDNA or its product, synthetic or otherwise, which can be transcribed into genomic or antigenomic RNA capable of serving as template to produce the genome of an infectious virus or subviral particle. Thus, defined mutations can be introduced by conventional techniques (e.g., site-directed
10   mutagenesis) into a cDNA copy of the genome or antigenome. The use of antigenome or genome cDNA subfragments to assemble a complete antigenome or genome cDNA as described herein has the advantage that each region can be manipulated separately (smaller cDNAs are easier to manipulate than large ones) and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or any
15   subfragment thereof can be used as template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the Muta-gene® kit of Bio-Rad Laboratories (Richmond, CA) or a method using a double-stranded plasmid directly as template such as the Chameleon mutagenesis kit of Stratagene (La Jolla, CA), or by the polymerase chain reaction employing either an
20   oligonucleotide primer or template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and available for use in producing the mutations of interest in the PIV antigenome or genome cDNA. Mutations can vary from single nucleotide changes to replacement of large cDNA pieces containing one or more
25   genes or genome regions.

Thus, in one illustrative embodiment mutations are introduced by using the Muta-gene phagemid *in vitro* mutagenesis kit available from Bio-Rad. In brief, cDNA encoding a portion of a PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations
30   are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome fragment is then amplified and the mutated piece is then reintroduced into the full-length genome or antigenome clone.

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Infectious PIV of the invention are produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode a PIV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins necessary to generate a transcribing, replicating nucleocapsid.  Among the viral proteins

5       useful for coexpression to yield infectious PIV are the major nucleocapsid protein (N) protein, nucleocapsid phosphoprotein (P), large (L) polymerase protein, fusion protein (F), hemagglutinin-neuraminidase glycoprotein (HN), and matrix (M) protein.  Also useful in this context are products of the C, D and V ORFs of PIV.

cDNAs encoding a PIV genome or antigenome are constructed for

10      intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV.  By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny PIV genome.  Preferably a cDNA is constructed which is a positive-sense version of the PIV genome corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of

15      hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid.

In some embodiments of the invention the genome or antigenome of a recombinant PIV (rPIV) need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious.  Further, the genes or

20      portions thereof may be provided by more than one polynucleotide molecule, i.e., a gene may be provided by complementation or the like from a separate nucleotide molecule.  In other embodiments, the PIV genome or antigenome encodes all functions necessary for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

25      By "recombinant PIV" is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced therefrom.  The recombinant expression system will employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements

30      having a regulatory role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

To produce infectious PIV from a cDNA-expressed PIV genome or antigenome, the genome or antigenome is coexpressed with those PIV N, P and L

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proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive

5    infection can be supplied by coexpression.

Synthesis of PIV antigenome or genome together with the above-mentioned viral proteins can also be achieved *in vitro* (cell-free), e.g., using a combined transcription-translation reaction, followed by transfection into cells. Alternatively, antigenome or genome RNA can be synthesized *in vitro* and transfected into cells

10   expressing PIV proteins.

In certain embodiments of the invention, complementing sequences encoding proteins necessary to generate a transcribing, replicating PIV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. Preferably, the helper virus can be distinguished phenotypically from the virus encoded

15   by the PIV cDNA. For example, it is desirable to provide monoclonal antibodies which react immunologically with the helper virus but not the virus encoded by the PIV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the

20   PIV cDNA to provide antigenic diversity from the helper virus, such as in the HN or F glycoprotein genes.

In alternate embodiments of the invention, the N, P, L and other desired PIV proteins are encoded by one or more non-viral expression vectors, which can be the same or separate from that which encodes the genome or antigenome. Additional

25   proteins may be included as desired, each encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome. Expression of the genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a promoter for T7 RNA polymerase, which in turn is supplied by infection,

30   transfection or transduction with an expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., Virology 210:202-205, 1995, incorporated herein by reference in its entirety). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells or by transfection of preformed mRNA or protein.

A PIV antigenome may be constructed for use in the present invention by, e.g., assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction or the like (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and *PCR Protocols: A Guide to Methods and*

5    *Applications*, Innis et al., eds., Academic Press, San Diego, 1990; each incorporated herein by reference in its entirety) of reverse-transcribed copies of PIV mRNA or genome RNA. For example, a first construct is generated which comprises cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a

10   plasmid, cosmid, phage, or DNA virus vector. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired PIV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking

15   ribozyme and tandem T7 transcriptional terminators. The ribozyme can be hammerhead type (e.g., Grosfeld et al., J. Virol. 69:5677-5686, 1995), which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., Nature 350:434-436, 1991), incorporated herein by reference in its entirety) which would yield a 3' end free of non-

20   PIV nucleotides. The left- and right-hand ends are then joined via a common restriction site.

A variety of nucleotide insertions, deletions and rearrangements can be made in the PIV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at

25   appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as site-specific mutagenesis, alanine scanning, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Alternative means to construct cDNA encoding the genome or antigenome

30   include reverse transcription-PCR using improved PCR conditions (e.g., as described in Cheng et al., Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994, incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (e.g., T3, SP6) or different

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ribozymes (e.g., that of hepatitis delta virus. Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome may be inserted into appropriate host cells by transfection, electroporation, mechanical

5    insertion, transduction or the like, into cells which are capable of supporting a productive PIV infection, e.g., HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb,

10   Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., New York, 1987), cationic lipid-mediated transfection (Hawley-Nelson et al., Focus 15:73-79, 1993) or a commercially available transfection regent, e.g., LipofectACE® (Life Technologies, Gaithersburg, MD) or the

15   like (each of the foregoing references are incorporated herein by reference in its entirety).

As noted above, in some embodiments of the invention the N, P, L and other desired PIV proteins are encoded by one or more helper viruses which is phenotypically distinguishable from that which encodes the genome or antigenome. The N, P, L and other desired PIV proteins can also be encoded by one or more expression

20   vectors which can be the same or separate from that which encodes the genome or antigenome, and various combinations thereof. Additional proteins may be included as desired, encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome.

By providing infectious clones of PIV the invention permits a wide range

25   of alterations to be recombinantly produced within the PIV genome (or antigenome), yielding defined mutations which specify desired phenotypic changes. By "infectious clone" is meant cDNA or its product, synthetic or otherwise, RNA capable of being directly incorporated into infectious virions which can be transcribed into genomic or antigenomic RNA capable of serving as a template to produce the genome of infectious

30   viral or subviral particles. As noted above, defined mutations can be introduced by a variety of conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA subjects provide for

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better ease of manipulation than large cDNA subjects, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using

5      the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or a template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome

10     cDNA. A variety of other mutagenesis techniques are known and can be routinely adapted for use in producing the mutations of interest in a PIV antigenome or genome cDNA of the invention.

       Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid in vitro mutagenesis kit available from Bio-Rad Laboratories.

15     In brief, cDNA encoding an PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is

20     then amplified.

       Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome segments can correspond to structural and/or functional domains, e.g., cytoplasmic, transmembrane or ectodomains of proteins, active sites such

25     as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, e.g., sites that stimulate antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small functional domains of proteins, e.g., epitopic sites, to about 50, 75, 100, 200-500, and 500-1,500 or more

30     nucleotides.

       The ability to introduce defined mutations into infectious PIV has many applications, including the manipulation of PIV pathogenic and immunogenic mechanisms. For example, the functions of PIV proteins, including the N, P, M, F, HN, and L proteins and C, D and V ORF products, can be manipulated by introducing

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mutations which ablate or reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the invention. The effects of trans-acting proteins and cis-acting RNA

5 sequences can be readily determined, for example, using a complete antigenome cDNA in parallel assays employing PIV minigenomes (Dimock et al., J. Virol. 67:2772-2778, 1993, incorporated herein by reference in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to be recovered in replication-independent infectious virus.

10             Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant PIV of the invention (e.g., substitutions of a genome segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) are made in structural or

15 functional relation to an existing, "counterpart" gene or genome segment from the same or different PIV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild-type or parental PIV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of

20 another PIV. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

            As used herein, "counterpart" genes, genome segments, proteins or protein regions, are typically from heterologous sources (e.g., from different PIV genes, or representing the same (i.e., homologous or allelic) gene or genome segment in different

25 PIV types or strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding genome segments embrace an assemblage of species having a range of size and sequence

30 variations defined by a common biological activity among the domain or genome segment variants.

            Counterpart genes and genome segments, as well as other polynucleotides disclosed herein for producing recombinant PIV within the invention, often share substantial sequence identity with a selected polynucleotide "reference sequence," e.g.,

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with another selected counterpart sequence. As used herein, a "reference sequence" is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length,

5 and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two

10 polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may

15 comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman (Adv. Appl. Math.

20 2:482, 1981; incorporated herein by reference), by the homology alignment algorithm of Needleman & Wunsch, (J. Mol. Biol. 48:443, 1970; incorporated herein by reference), by the search for similarity method of Pearson & Lipman, (Proc. Natl. Acad. Sci. USA 85:2444, 1988; incorporated herein by reference), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics

25 Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI, incorporated herein by reference), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the

30 window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e.,

the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity,

5      more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the

10     window of comparison. The reference sequence may be a subset of a larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein regions encoded by recombinant PIV of the invention are also typically selected to have conservative relationships, i.e. to have substantial sequence identity or sequence similarity, with selected reference polypeptides. As applied to polypeptides, the term

15     "sequence identity" means peptides share identical amino acids at corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (i.e., conservative substitutions) at corresponding positions. The term "substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent

20     sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions

25     refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine,

30     and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used herein

follow conventional usage (Immunology - A Synthesis, 2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates, Sunderland, MA, 1991, incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and

5      other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3 -methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-

10     hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

           To select candidate vaccine viruses according to the invention, the criteria of viability, attenuation and immunogenicity are determined according to well known methods. Viruses which will be most desired in vaccines of the invention must maintain

15     viability, have a stable attenuation phenotype, exhibit replication in an immunized host (albeit at lower levels), and effectively elicit production of an immune response in a vaccinee sufficient to confer protection against serious disease caused by subsequent infection from wild-type virus. The recombinant PIV of the invention are not only viable and more appropriately attenuated than previous vaccine candidates, but are more stable

20     genetically *in vivo*--retaining the ability to stimulate a protective immune response and in some instances to expand the protection afforded by multiple modifications, e.g., induce protection against different viral strains or subgroups, or protection by a different immunologic basis, e.g., secretory versus serum immunoglobulins, cellular immunity, and the like.

25            Recombinant PIV of the invention can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus (e.g., a multiply attenuated, biologically derived or recombinant PIV) is tested, e.g., level of replication, for temperature sensitivity of virus replication, i.e. ts

30     phenotype, and for the small plaque or other desired phenotype. Modified viruses are further tested in animal models of PIV infection. A variety of animal models have been described and are summarized in various references incorporated herein. PIV model systems, including rodents and non-human primates, for evaluating attenuation and

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immunogenic activity of PIV vaccine candidates are widely accepted in the art, and the data obtained therefrom correlate well with PIV infection, attenuation and immunogenicity in humans.

In accordance with the foregoing description, the invention also provides

5      isolated, infectious recombinant PIV viral compositions for vaccine use. The attenuated virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to PIV which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial

10     medium where it can be propagated and characterized in a controlled setting. For example, attenuated PIV of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

For vaccine use, recombinant PIV produced according to the present invention can be used directly in vaccine formulations, or lyophilized, as desired, using

15     lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, $Mg^{++}$ and HEPES, with or without adjuvant, as further described below.

PIV vaccines of the invention contain as an active ingredient an

20     immunogenically effective amount of PIV produced as described herein. The modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being

25     combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan

30     monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, MPL™ (3-o-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

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Upon immunization with a PIV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for PIV virus proteins, e.g., F and HN glycoproteins. As a result of the vaccination with an immunogenically effective amount

5 of PIV produced as described herein, the host becomes at least partially or completely immune to PIV infection, or resistant to developing moderate or severe PIV infection, particularly of the lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by PIV or a closely related virus and which host is

10 capable of generating a protective immune response to the antigens of the vaccinizing strain. Accordingly, the invention provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the PIV of the invention are administered to a host susceptible to or otherwise at risk for PIV infection to enhance the

15 host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of PIV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about $10^3$ to about $10^7$ plaque forming units (PFU) or more of virus per host, more

20 commonly from about $10^4$ to $10^6$ PFU virus per host. In any event, the vaccine formulations should provide a quantity of modified PIV of the invention sufficient to effectively protect the host patient against serious or life-threatening PIV infection.

The PIV produced in accordance with the present invention can be combined with viruses of other PIV serotypes or strains to achieve protection against

25 multiple PIV serotypes or strains. Alternatively, protection against multiple PIV serotypes or strains can be achieved by combining protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered simultaneously, but they may also be administered separately. Immunization with one

30 strain may protect against different strains of the same or different serotype.

In some instances it may be desirable to combine the PIV vaccines of the invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. In another aspect of the invention the PIV can be employed as a vector for protective antigens of other pathogens, such as respiratory syncytial virus

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(RSV) or measles virus, by incorporating the sequences encoding those protective antigens into the PIV genome or antigenome which is used to produce infectious PIV, as described herein (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference).

5        In all subjects, the precise amount of recombinant PIV vaccine administered, and the timing and repetition of administration, will be determined based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about $10^3$ to about $10^7$ plaque forming units (PFU) or more of virus per patient, more commonly from about $10^4$ to $10^6$
10   PFU virus per patient. In any event, the vaccine formulations should provide a quantity of attenuated PIV sufficient to effectively stimulate or induce an anti-PIV immune response, e.g., as can be determined by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As
15   with administration to rhesus monkeys, the attenuated virus of the vaccine grows in the nasopharynx of vaccinees at levels approximately 10-fold or more lower than wild-type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated PIV.

       In neonates and infants, multiple administration may be required to elicit
20   sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) PIV infection. Similarly, adults who are particularly susceptible to repeated or serious PIV infection, such as, for example, health care workers, day care workers, family
25   members of young children, the elderly, individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine
30   viruses may be indicated for administration to different recipient groups. For example, an engineered PIV strain expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

       PIV vaccines produced in accordance with the present invention can be combined with viruses expressing antigens of another subgroup or strain of PIV to

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achieve protection against multiple PIV subgroups or strains. Alternatively, the vaccine virus may incorporate protective epitopes of multiple PIV strains or subgroups engineered into one PIV clone, as described herein.

5      The PIV vaccines of the invention elicit production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild-type PIV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination and possible boosting of resistance by subsequent infection by wild-type

10     virus. Following vaccination, there are detectable levels of host-engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus *in vitro* and *in vivo*. In many instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup.

Preferred PIV vaccine candidates of the invention exhibit a very

15     substantial diminuition of virulence when compared to wild-type virus that is circulating naturally in humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus may still be capable of dissemination to unvaccinated individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the

20     vaccinated or incidental host do not occur.

The level of attenuation of PIV vaccine candidates may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type PIV or other attenuated PIV which have been evaluated as candidate vaccine strains. For example, the

25     attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, or rhesus monkey, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus

30     should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals. Methods for determining levels of PIV in the nasopharynx of an infected host are well known in the literature.

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Levels of induced immunity provided by the vaccines of the invention can also be monitored by measuring amounts of neutralizing secretory and serum antibodies. Based on these measurements, vaccine dosages can be adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses

5       may be advantageous for different recipient groups. For example, an engineered PIV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

In yet another aspect of the invention the PIV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the

10      recombinant PIV genome or antigenome incorporates a sequence which is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls PIV expression. The infectious PIV produced by coexpressing the recombinant PIV genome or antigenome with the N, P, L and other desired PIV proteins, and containing a sequence encoding the gene product of

15      interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant PIV is administered in an amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Representative gene products which may be administered within this method are preferably suitable for transient expression,

20      including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and vaccine antigens.

25      The following examples are provided by way of illustration, not limitation.


## EXAMPLE I

### Construction of cDNAs Encoding a Chimeric HPIV3/BPIV3 Antigenome and Recovery of Infectious Virus

30      The following three examples document studies to identify which of the proteins of BPIV3 contribute to its host range restriction in primates. To illustrate these methods, the N protein of the wild type HPIV3 virus was replaced with its counterpart from BPIV3. This exchange was accomplished using a reverse genetics system for recovery of infectious PIV from cDNA as described above. The studies were initiated

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with the N gene of BPIV3 because this protein possesses an intermediate level of amino acid sequence difference from its HPIV3 counterpart compared to other HPIV3 and BPIV3 proteins (see Example I).

5    A chimeric recombinant virus was constructed in which the N ORF of the JS strain of HPIV3 was replaced by that of either the Ka or SF strain of BPIV3 (Fig. 1). These chimeric viruses possess the HN and F glycoproteins of the HPIV3 parent and will induce a high level of immunity to HPIV3 in primates. Both chimeric viruses were successfully recovered. Both grew to high titer in cell culture and both were found to be attenuated in rhesus monkeys. Thus, the N protein was identified as an exemplary protein

10   that contributes to the host range phenotype of BPIV3. Immunization of rhesus monkeys with either the Ka or SF chimeric recombinant virus induced a high level of resistance to the replication of HPIV3 used as a wild type challenge.

The present invention, therefore, establishes the usefulness of reverse genetics methods to generate chimeric human-bovine PIV virus that combines the host

15   range attenuation properties of BPIV3 and the immunogenicity of the HPIV3 HN and F protective antigens. Immunization of humans with such a chimeric recombinant will redress the problem of suboptimal immunogenicity of the BPIV3 vaccine previously observed in humans.

The complete consensus nucleotide sequence for each of the Ka or SF

20   BPIV3 strains was determined from RT-PCR products generated from virion RNA. These sequences are set forth in Figures 6A-6G, and Figures 7A-7G, respectively. The full length cDNA encoding a complete 15456 nucleotide (nt) antigenomic RNA of BPIV3 Ka is set forth in Figures 6A-6G herein (see also GenBank accession #AF178654). The GenBank sequence for BPIV3 kansas strain differs from the sequence of the exemplary

25   cDNA in two positions at nucleotide 21 and 23. Both, the published sequence and the sequence in the exemplary cDNA occur naturally in kansas strain virus population with similar frequencies. The cDNA used in the present example contains a sequence beginning at nucleotide 18, ACT_GG_TT (SEQ ID NO. 1), whereas the corresponding published sequence (GenBank accession #AF178654; Figures 6A-6G, SEQ ID NO. 22)

30   reads ACT_T_GC_T (differing nucleotides at positions 21 and 23 are underscored).

To construct consensus nucleotide sequences for the Ka and SF BPIV3 strains, virion RNA was subjected to reverse transcription using the Superscript II Preamplification System (Life Technologies, Gaithersburg, MD) and 200 ng of random hexamer primers. PCR was carried out on the first strand product using the Advantage

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cDNA PCR kit (Clontech Laboratories, Palo Alto, CA). Ka and SF genomes were each amplified by PCR in 3 or 4 overlapping fragments using primers homologous to regions of RNA conserved among previously-published paramyxovirus sequences. Each primer pair was constructed to include matching restriction enzyme sites (not represented in the

5     sequence targeted for amplification).

A separate random library was generated for each amplicon by digesting a set of PCR products with the appropriate restriction enzyme, followed by gel-purification, ligation of the products into tandem arrays and sonication. A random library was generated from this pool of sheared cDNA sequences by cloning a subset (approx. 500 bp

10   fragments) into M 13. The nucleotide sequences of cDNA inserts were determined by automated DNA sequencing using the Taq DYE Deoxy Terminator cycle sequencing kit (ABI, Foster City, CA). A continuous sequence (contig) was assembled for each of the original large RT-PCR fragments with sufficient redundancy that each nucleotide position was confirmed by a minimum 3 independent M13 clones. The 5' and 3' terminal

15   genomic sequences of Ka and SF were converted to cDNA using the system for Rapid Amplification of cDNA Ends (Life Technologies, Gaithersburg, MD) and sequenced by automated sequencing.

These sequences are set forth in Figures 6A-6G (Ka) and Figures 7A-7G (SF), respectively. Analysis of these sequences revealed that the percent amino acid

20   identity between HPIV3 and BPIV3 for each of the following proteins is: N (86%), P (65%), M (93%), F (83%), HN (77%), and L (9 1%). Thus sequence divergence was found distributed over many genes. The deduced amino acid sequence of the N genes of these two viruses is presented in GenBank #Af178654 (Ka) and #AF178655 (SF), not included. The position of the N ORF in the BPIV3 genome is indicated in the respective

25   BenBank reports and included herein by reference. In the example below, the N ORF of the Ka or SF virus was initially selected for replacement of the corresponding gene in the HPIV3 virus because the N gene represents a gene with an intermediate level of sequence divergence among the six HPIV3 and BPIV3 proteins. In this study the N ORF, but not the 3' or 5, noncoding N gene sequences, was exchanged, which permitted us to assign an

30   observed attenuation phenotype of cKa and cSF to the protein encoded by the N gene.

Human-bovine chimeric full-length PIV3 genomes were constructed by introducing the BPIV3 Ka or SF N coding region as a replacement for its HPIV3 counterpart into the rJS cDNA p3/7(131)2G which encodes a complete copy of HPIV3 positive-sense antigenomic RNA (see, e.g., Durbin et al., 1997a, *supra*; Hoffman et al.,

1997, *supra*; Skiadopoulos et al., 1998, *supra*; U.S. Patent Application Serial No.
09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May
23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S.
Provisional Application No. 60/059,385, filed September 19, 1997; each incorporated

5      herein by reference). BPIV3 and HPIV3 N coding regions with flanking sequences were
first subcloned and further modified to permit an exchange of just the N ORF.
pUC119JSN bearing the HPIV3 N gene and the plasmids with a BPIV3 N Ka or SF gene
(pBSKaN and pBSSFN) were subjected to mutagenesis using the method of Kunkel
(Proc. Natl. Acad. Sci. USA 82:488-492, 1985, incorporated herein by reference) to

10     introduce Ncol and AflII restriction enzyme recognition sites at translational start and
stop sites, respectively (Fig. 1A). Following Ncol/AflII digestion of pUC119KaN-
Ncol/AflII, the BPIV3 N coding region was introduced as an Ncol/AfllII fragment into
pUC119JSN-Ncol/AflII as a replacement for the HPIV3 N coding region (Fig. 1 B). The
chimeric N genes, which contain the HPIV3 3' and 5' noncoding sequences and the

15     BPIV3 ORF, were modified by site-directed mutagensis to restore the original HPIV3
noncoding sequence and BPIV3 coding sequence. This chimeric N gene was then
introduced into the 5' half of the rJS antigenome, pLeft, in exchange for its corresponding
HPIV3 sequence (Figs. 2A and 2B) using existing MIuI and EcoRI sites present in the
human sequence. In each case parallel reactions were carried out for the SF N ORF. The

20     chimeric pLeft plasmid was combined with the XhoI/NgoMI fragment from pRight
containing the 3' half of the rJS antigenome flanked by the delta ribozyme and the T7
terminator at its 3' end (Fig. 2). The resulting chimeric PIV3 plasmids designated
pB/HPIV3NKa or pB/HPIV3NSF, contained the full-length rJS antigenome in which the
N ORF encoded the BPIV3 Ka or SF N protein.

25             Chimeric antigenomic HPIV3/BPIV3 cDNAs were transfected
individually into HEp-2 cells grown to near-confluence in 6-well plates along with two
previously-described support plasmids, pTM(P no C) and pTM(L), Lipofectace (Life
Technologies, Gaithersburg, MD), and infected with a modified vaccinia virus
recombinant that expresses bacteriophage T7 RNA polymerase (MVA-T7) as previously

30     described (Durbin et al., Virology 234:74-83, 1997b). An N support plasmid used in
previous work was omitted because the antigenomic plasmid expressed sufficient levels
of the N protein. The cultures were maintained for 3.5 days at 32°C after which
supernatants were harvested, passaged in LLC-MK2 cells and plaque-purified 3 times in

LLC-MK2 cells. The identities of the chimeric viruses incorporating a human PIV 3 background genome or antigenome and a BPIV3 N protein (designated as rHPIV3-N_B chimeric recombinants or, more specifically, as "cKa" and "cSF" chimeric viruses) recovered from the transfections were confirmed by sequencing RT-PCR products

5   containing the regions of the N ORF start and stop codons from virion RNA isolated after amplification of triply plaque-purified virus (Fig. 3). This amplified product and the corresponding amplified HPIV3 rJS and BPIV3 Ka or SF sequences were also subjected to TaqI digestion to confirm the chimeric identity of cKa and cSF viruses (Fig. 4). TaqI digestion profiles were distinct for the 3 parental and 2 chimeric viruses, and each

10  parental profile included TaqI fragments of unique size, allowing the contribution of sequence of rJS, Ka and SF parents to the chimeric viruses to be verified. The recovered cKa and cSF chimeric recombinants each contained the expected sequences as designed.


## EXAMPLE II

15  ### Replication of HPIV3/BPIV3 Chimeric Viruses in Cell Culture

Efficient replication of live attenuated virus vaccines in tissue culture cells is a feature of human-bovine chimeric PIV of the invention that permits efficient manufacture of the recombinant vaccine materials. The multicycle replication of rJS parent, cKa, Ka parent, cSF, and SF parent in a bovine cell line (MDBK) and in a simian

20  cell line (LLC-MK2) was determined by infecting cells with virus at a multiplicity of infection of 0.01 and harvesting samples (in triplicate) over a five day period of time (Fig. 5) as previously described (Tao et al., 1998, *supra*, incorporated herein by reference). The chimeric viruses replicated efficiently in both cell lines like their human or bovine parent viruses without significant delay in replication or a significant reduction in the titer

25  of virus achieved. In each case, the chimeric viruses replicated to over $10^{7.0}$ TCID_{50}/ml which is well above the $10^{4.0}$ or $10^{5.0}$ dose of live attenuated human or bovine PIV vaccines currently being used in human clinical trials (Karron et al., 1996, *supra*; Karron et al., 1995a, *supra*; and Karron et al., 1995b, *supra*).


30  ## EXAMPLE III

### Evaluation of Attenuation and Protective Efficacy of the HPIV3/BPIV3 Chimeric Viruses in Rhesus Monkeys

Both the SF and Ka BPIV3s are attenuated for the upper and the lower respiratory tract of the rhesus monkey (van Wyke Coelingh et al., 1988, *supra*). This

attenuation phenotype correlates with attenuation in humans (Karron et al., 1995a, *supra*) as indicated by the fact that Ka is highly restricted in replication in the upper respiratory tract of fully susceptible seronegative infants and children. The absence of cough, croup, bronchiolitis, or pneumonia in the BPIV3-infected vaccinees suggests that the Ka BPIV3

5    virus is attenuated for the lower respiratory tract as well. Therefore, the rhesus monkey is widely accepted as a reasonably correlative model to evaluate attenuation of candidate PIV vaccine viruses and their efficacy against challenge with wild type PIV.

The rJS, cKa, Ka parent, cSF, and SF parent were administered intranasally and intratracheally at a dose of $10^{5.0}$ TCID$_{50}$ per site to rhesus monkeys.

10   Replication was monitored using previously described procedures for obtaining samples from the upper (nasopharyngeal swab specimens) and lower (tracheal lavage specimens) respiratory tract and for titering the virus in LLC-MK2 cells (Hall et al., 1992, *supra*). The cKa and cSF recombinants were significantly attenuated for the upper respiratory tract (Table 1) exhibiting, respectively, a 63-fold or a 32-fold reduction in mean peak

15   virus titer compared to that of the rJS HPIV3 parent. Both cKa and cSF were also attenuated for the lower respiratory tract, but this difference was only statistically significant for cSF. The low level of replication of rJS in the lower respiratory tract made it difficult to demonstrate in a statistically-significant fashion further restriction of replication due to an attenuation phenotype at this site.

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Table 1. Replication of cKa and cSFis restricted relative to HPIV3 in the upper and lower respiratory tracts of rhesus monkeys.

Virus Replication

| Immu-nizing virus[1] | No. of animals | Mean titers $Log_{10}TCID_{50}/ml\pm$standard error [Duncan grouping][2] | | | | Mean peak titers $Log_{10}TCID_{50}/ml\pm$standard error [Duncan grouping] | |
|---|---|---|---|---|---|---|---|
| | | Nasopharynx | | Trachea | | Nasopharynx | Trachea |
| | | day 6 | day 7 | day 4 | day 6 | | |
| rJS | 4 | 5.3±0.59[A] | 3.9±0.36[A] | 1.7±0.45[A] | 1.7±0.29[A] | 5.3±0.59[A] | 2.5±50.51[A] |
| cKa | 4 | 3.0±0.58[B] | 2.9±0.42[AB] | 1.5±0.40[A] | 1.0±0.19[A] | 3.5±0.54[B] | 1.5±0.18[AB] |
| Ka | 4 | 2.0±0.27[B] | 2.4±0.30[B] | 1.3±0.26[A] | 1.3±0.21[A] | 2.5±0.30[B] | 1.6±0.15[AB] |
| cSF | 4 | 3.3±0.40[B] | 3.7±0.57[A] | 1.1±0.25[A] | 1.1±0.24[A] | 3.8±0.46[B] | 1.4±0.26[B] |
| SF | 4 | 2.8±0.48[B] | 2.6±0.40[AB] | 1.6±0.46[A] | 1.5±0.40[A] | 3.3±0.28[B] | 1.8±0.41[AB] |

[1] Monkeys were inoculated intranasally and intratracheally with $10^{5.0}$ $TCID^{50}$ in 1 ml at each site.

[2] Mean viral titers in each column were assigned to statistically similar groups (designated with a letter) using a Duncan Multiple Range test ($\alpha = 0.05$). Mean titers in each column with different letters are statistically different.

The level of replication of each chimeric virus, cKa and cSF, was not significantly different from its bovine parent in the upper or the lower respiratory tract, although the chimeric viruses each replicated somewhat better than their BPIV3 parents in the upper respiratory tract. Thus, the acquisition of the N gene of either the Ka or SF

5   BPIV3 by rJS HPIV3 attenuated the human virus for rhesus monkeys to a level approximately equivalent to that of the BPIV parent. Since the HPIV3/BPIV3 chimeric recombinants replicated efficiently in tissue culture cells *in vitro*, it is clear that the phenotype of host range restricted replication manifested by the two bovine parental viruses was transferred to HPIV3 by the N ORF. It is possible, but unknown and

10   unpredictable, that substitution of other BPIV3 genes, such as M, P, or L, for their HPIV3 counterpart in rJS will give similar or greater levels of attenuation as observed upon substitution of the BPIV3 N gene for the HPIV3 N gene. The observation that the level of replication of cKa and cSF is slightly greater than that of their BPIV3 parents in the upper respiratory tract suggests that additional bovine genes contribute to the host range

15   attenuation phenotype of the BPIV3 parent virus at this site.

Uninoculated monkeys and monkeys that were previously infected with a human or bovine PIV3 parental virus, or with the cKa or cSF chimeric virus, were challenged 42 days after the initial inoculation with $10^{6.0}$ TCID$_{50}$ of rJS intranasally and intratracheally in a 1 ml inoculum at each site. The nasopharynx and the trachea were

20   sampled as described previously on the days indicated in Table 2. The titer of virus present at each site was determined for each monkey on LLC-MK2 cell monolayers, and the titers presented are mean peak titers (Hall et al., 1992, *supra*). Previous infection with either chimeric virus induced a high level of resistance to replication of the rJS challenge virus in both the upper and lower respiratory tract. Monkeys previously infected with

25   cKa manifested a 300-fold reduction of replication of wild type HPIV3 (rJS) in the upper respiratory tract and a 1000-fold reduction in the lower tract compared to uninoculated control monkeys. Monkeys previously infected with cSF manifested a 2000-fold reduction of replication of rJS in the upper respiratory tract and a 1000-fold reduction in the lower tract compared to uninoculated control monkeys. The level of reduction of

30   replication of rJS challenge virus in monkeys previously-inoculated with cKa or cSF was comparable to that of monkeys previously infected with either the bovine or the human PIV parent. Thus, infection with either HPIV3/BPIV3 chimeric virus provided a high level of protection in the upper and lower respiratory tract of monkeys, and both chimeric viruses represent promising vaccine candidates.

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Serum collected from monkeys on days 0 and 28 was tested by HAI assay
using HPIV3 (JS strain) and BPIV3 (Ka strain) as antigen as previously described
(Coelingh et al., J. Infect. Dis. 157:655-662, 1988). Although cKa-N and cSF-N were
5    highly attenuated in the upper and lower respiratory tract of rhesus monkeys relative to
rJS, each chimeric virus induced a hemagglutination-inhibiting (HAI) antibody response
to HPIV3 that was 2.5 to 5-fold greater in magnitude than that induced by immunization
with its respective BPIV3 patent. This likely is due to the presence of HPIV3 HN protein
in the chimeric viruses. Furthermore, the HPIV3-specific HAI-responses induced by the
10   chimeric viruses were statistically indistinguishable from that induced by immunization
with rJS. An additional unexpected result demonstrated herein is that, following
challenge of the monkeys with HPIV3, the level of HAI antibody in monkeys initially
immunized with cKa-N or cSF-N was significantly greater than levels observed in
animals immunized with rJS, Ka or SF.

## EXAMPLE IV

### Construction and Characterization of Chimeric HPIV3/BPIV3 Vaccine Candidates Having Heterologous Fusion And Hemagglutinin-Neuraminidase Glycoproteins

5          In the preceding example, the basis of host range restriction of replication
of BPIV3 for the respiratory tract of primates was examined by the generation and
characterization of a recombinant human PIV3 (rHPIV3) in which the N open reading
frame (ORF) was replaced by that of its BPIV3 counterpart. The resulting chimeric virus,
rHPIV3-$N_B$, also referred to as cKa or cSF, efficiently replicated in vitro but was
10        restricted in replication in the upper respiratory tract of rhesus monkeys, identifying the N
protein as an independent determinant of the host range restriction of BPIV3 in rhesus
monkeys (Bailly et al., J. Virol. 74:3188-3195, 2000).

          In the present example, the contribution of the fusion (F) and
hemagglutinin-neuraminidase (HN) glycoprotein genes of bovine parainfluenza virus type
15        3 (BPIV3) to its restricted replication in the respiratory tract of non-human primates was
examined by generating and characterizing two reciprocal chimeric BPIV3/HPIV3
viruses. A chimeric HPIV3 containing heterologous BPIV3 F and HN glycoprotein genes
in place of its own, and the reciprocal recombinant comprising a BPIV3 "backbone"
bearing the HPIV3 F and HN genes substituted for the counterpart BPIV3 glycoprotein
20        genes, were generated to assess the effect of glycoprotein substitution on replication of
HPIV3 and BPIV3 in the upper and lower respiratory tract of rhesus monkeys. Thus, in
one chimeric virus, the F and HN genes of HPIV3 were replaced with their BPIV3
counterparts, resulting in a chimeric recombinant designated rHPIV3-$F_BHN_B$. The
reciprocal chimeric recombinant PIV3 (rBPIV3-$F_HHN_H$) was constructed by replacing the
25        F and HN genes of a recombinant BPIV3 (rBPIV3) with their HPIV3 counterparts. In the
latter virus, the introduction of the HPIV3 F and HN ORFs into the BPIV3 backbone
combines the antigenic determinants of HPIV3 with the backbone of BPIV3 and thus
provides an improved vaccine candidate compared with parental BPIV3. The F and HN
genes were exchanged as pairs in view of the proposed requirement for homologous HN
30        and F proteins for parainfluenza viruses for full functional activity (Deng et al., Virology
209:457-469, 1995; and Tanabayashi et al., J. Virol. 70:6112-6118, 1996; each
incorporated herein by reference).

          The foregoing chimeric viruses were readily recovered and exhibited
kinetics of replication in simian LLC-MK2 cells that were comparable to those of their

parent viruses, suggesting that the heterologous glycoproteins were compatible with the
PIV3 internal proteins. The distinctive features of cytopathology of BPIV3 versus HPIV3
cosegregated with their respective F and HN genes. HPIV3 bearing the BPIV3 F and HN
genes was attenuated for replication in rhesus monkeys to a level similar to that of its

5      BPIV3 parent virus, indicating that the glycoprotein genes of BPIV3 are major
determinants of its host range restriction of replication in rhesus monkeys. BPIV3
bearing the HPIV3 F and HN genes (rBPIV3-$F_HHN_H$) replicated in rhesus monkeys to a
level intermediate between that of HPIV3 and BPIV3.

These results indicate that the F and HN genes make a significant

10     contribution to the overall attenuation of BPIV3. Furthermore, they demonstrate that
BPIV3 sequences outside the F and HN region also contribute to the attenuation
phenotype in primates. This latter finding is consistent with the demonstration in the
preceding example that the nucleoprotein coding sequence of BPIV3 is a determinant of
its attenuation for primates. Despite its restricted replication in the respiratory tract of

15     rhesus monkeys, rBPIV3-$F_HHN_H$ conferred a level of protection against challenge with
wild type HPIV3 that was indistinguishable from that conferred by previous infection
with wild type HPIV3. From these and related findings, the usefulness of rBPIV3-
$F_HHN_H$ as a vaccine candidate against HPIV3 is readily apparent.

20     **Viruses and cells**

HEp-2 and simian LLC-MK2 monolayer cell cultures were maintained in
MEM medium (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal
bovine serum (Summit Biotechnology, Ft. Collins, CO), 50ug/ml gentamicin sulfate, and
4mM glutamine (Life Technologies, Gaithersburg, MD).

25     The wild type BPIV3 strain Kansas/15626/84 (Clone 5-2-4, Lot BPI3-1)
(BPIV3 Ka), the HPIV3 JS wild type, its recombinant version (rHPIV3), and the rHPIV3
virus containing the BPIV3 Ka N ORF in place of the HPIV3-N ORF (rHPIV3-$N_B$) are
each described above (see also, Clements et al., 1991, *supra*; Karron et al., 1995a, *supra*;
Bailly et al., 2000, *supra*; and Durbin et al., 1997, *supra*). PIVs were propagated at 32°C

30     in LLC-MK2 cells (ATCC CCL-7), as previously described (Hall et al., 1992, *supra*).
The modified vaccinia strain Ankara (MVA) recombinant virus that expresses
bacteriophage T7 RNA polymerase is described by Wyatt et al. (1995, *supra*).

**Construction of antigenomic cDNAs encoding recombinant BPIV3/HPIV3 viruses.**

a) <u>Construction of cDNA to recover rBPIV3</u>

A full length cDNA was constructed to encode the complete 15456 nucleotide (nt) antigenomic RNA of BPIV3 Ka, as described above. The cDNA was

5      assembled from 4 subclones derived from reverse transcription (RT) of viral RNA using the SuperScript II Pre-amplification System (Life Technologies, Gaithersburg, MD) and polymerase chain reaction (PCR) amplification with a High Fidelity PCR kit (Clontech Laboratories, Palo Alto, CA). The RT-PCR products were cloned into modified pUC19 plasmids (New England Biolabs, Beverly, MA) using the following naturally occuring

10     internal restriction enzyme recognition sites: *Sma* I (BPIV3 Ka sequence position nt186), *Pst* I (nt 2896), *Mlu* I (nt 6192), *Sac* II (nt 10452) and *Bsp* LU11 (nt 15412). Multiple subclones of the antigenomic cDNA were sequenced using a Perkin Elmer ABI 310 sequencer with dRhodamine Terminator Cycle Sequencing (Perkin Elmer Applied Biosystems, Warrington, UK), and only those matching the consensus sequence of BPIV3

15     Ka were used for assembly of the full length clone. The 3' and 5' ends of BPIV3 Ka were cloned and the assembly of the full length cDNA took place in the previously described p(Right) vector (Durbin et al., 1997, *supra*), which we modified to contain a new polylinker with restriction enzyme recognition sites for *Xho* I, *Sma* I, *Mlu* I, *Sac* II, *Eco* RI, *Hind* III and *Rsr*II. The full length cDNA clone pBPIV3(184) contained the

20     following elements in 3' to 5' order: a T7 promoter followed by 2 non-viral guanosine residues, the complete antigenomic sequence of BPIV3 Ka, a hepatitis delta virus ribozyme and a T7 polymerase transcription terminator (Bailly et al., 2000, *supra*; and Durbin et al., 1997a, *supra*).

25     b) <u>Construction of rHPIV3-F$_B$HN$_B$ and rBPIV3-F$_H$HN$_H$</u>

Unique restriction enzyme recognition sites were introduced into the BPIV3 antigenomic cDNA and into the previously described HPIV3 antigenomic cDNA p3/7(131)2G (Durbin et al., 1997a, *supra*) to facilitate the exchange of the F and HN genes between BPIV3 and HPIV3 cDNAs. Using the transformer site-directed

30     mutagenesis protocol from Clontech (Clontech Laboratories, Palo Alto, CA), *Sgr*AI restriction sites were introduced in the downstream non-coding region of the M gene at position 4811 of the rBPIV3 sequence and position 4835 of the rHPIV3 JS sequence (GenBank accession # Z11575). The nucleotide number given for the position of restriction enzyme recognition sites indicates the nucleotide after which the enzyme cuts,

not the first nucleotide of the restriction enzyme recognition site. The sequence was changed from TCCAACATTGCA (SEQ. ID. NO. 2) to TCCACCGGTGCA (SEQ. ID. NO. 3) in rBPIV3 and from CGGACGTATCTA (SEQ. ID. NO. 4) to CGCACCGGTGTA (SEQ. ID. NO. 5) in rHPIV3 (recognition sites underlined). *Bsi*WI restriction sites were introduced in the downstream non-coding region of the HN gene at nt 8595 of the rBPIV3 sequence and at nt 8601 of the rHPIV3 JS sequence. The sequence was changed from GATATAAAGA (SEQ. ID. NO. 6) to GACGTACGGA (SEQ. ID. NO. 7) in rBPIV3 to give pBPIVs(107) and from GACAAAAGGG (SEQ. ID. NO. 8) to GACGTACGGG (SEQ. ID. NO. 9) in rHPIV3 to give pHPIVs(106). The F and HN genes were exchanged between pBPIVs(107) and pHPIV3s(106) by digestion of each with *Sgr*AI and *Bsi*WI, gel purification of the fragments, and assembly of the appropriate fragments into the two full length cDNAs. The HPIV3 backbone bearing the BPIV3 F and HN genes, designated pHPIV(215), encoded 15480 nts of viral sequence, of which nts 4835 to 8619 came from BPIV3, and it was used to derive rHPIV3-$F_B HN_B$ (Figures 8A-8C). The BPIV3 backbone bearing the HPIV3 F and HN genes, designated pBPIV(215), encoded 15438 nts of viral sequence, of which nts 4811 to 8577 came from HPIV3, and it was used to derive rBPIV3-$F_H HN_H$ (Figures 8A-8C).

**BPIV3 support plasmids for recovery of virus from cDNA.**

Support plasmids encoding the BPIV3 Ka N, P and L genes were assembled in modified pUC19 vectors and then cloned into the previously described pTM-1 vector (Durbin et al., 1997a, *supra*). In order to place the individual genes immediately downstream of the T7 promoter in the pTM vector, an *Nco* I site was introduced at the start codon of the N, P and L open reading frames (ORFs) using site-directed mutagenesis. The *Nco* I restriction site and a naturally occurring restriction site downstream of each ORF (*Spe* I for N, *Hinc*II for P and *Bsp* LU11I for L) was used for cloning into pTM. After cloning, the *Nco* I site in pTM(N) was mutagenized back to the original sequence to restore the correct amino acid assignment in the second codon. In pTM(P) and pTM(L) the amino acid sequence encoded by the ORF was not altered by the introduction of *Nco* I sites.

**Transfection.**

HEp-2 cells (approximately $1.5 \times 10^6$ cells per well of a six-well plate) were grown to 90% confluence and transfected with 0.2 µg each of the BPIV3 support

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plasmids pTM(N) and pTM(P), and 0.1 µg of pTM(L), along with 5 µg of the full length antigenomic cDNA and 12µl LipofectACE (Life Technologies, Gaithersburg, MD). Each transfection mixture also contained $1.5 \times 10^7$ plaque forming units (PFU) of MVA-T7, as previously described (Durbin et al., 1997, *supra*). The cultures were incubated at 32°C

5    for 12 hrs before the medium was replaced with MEM (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum. The supernatants were harvested after incubation at 32°C for an additional three days, and were passaged onto LLC-MK2 cell monolayers in 25cm² flasks and incubated for 5 days at 32°C. Virus present in the supernatant was plaque-purified three times prior to amplification and characterization.

10

**Molecular characterization of recovered chimeric recombinants.**

The presence of the heterologous F and HN genes in the bovine or human PIV3 backbone was confirmed in plaque-purified recombinant viruses by RT-PCR of viral RNA isolated from infected cells or supernatant, which was performed using a

15    primer pair that recognizes conserved sequences in rBPIV3 and rHPIV3. This yielded similarly sized fragments (nts 4206-9035 in rBPIV3, nts 4224-9041 in rHPIV3, nts 4206-9017 in rBPIV3-$F_H$HN$_H$, and nts 4224-9059 in rHPIV3-$F_B$HN$_B$) which were then digested with *Eco* RI and analyzed by electrophoresis on a 1% agarose gel (Fig. 9). The nucleotide sequence flanking the introduced *Sgr*AI and *Bsi*WI restriction sites in each

20    virus was confirmed by sequencing the respective RT-PCR product.


**Replication of HPIV3/BPIV3 chimeric viruses in cell culture.**

The multicycle growth kinetics of BPIV3 Ka, rHPIV3-$F_B$HN$_B$, rBPIV3-$F_H$HN$_{II}$, rHPIV3-$N_B$ and rHPIV3 in LLC-MK2 cells were determined by infecting cells in

25    triplicate at a multiplicity of infection (MOI) of 0.01 and harvesting samples at 24 hr intervals over a six day period, as previously described (Tao et al., 1998, *supra*). Samples were flash-frozen and titered in a single assay on LLC-MK2 cell monolayers in 96 well plates at 32°C, as described (Durbin et al., Virology 261:319-330, 1999b, incorporated herein by reference).

30

**Primate Model Studies.**

Rhesus monkeys seronegative for PIV3 as determined by hemagglutination-inhibition (HAI) assay (van Wyke Coelingh et al., 1988, *supra*) were

inoculated intranasally and intratracheally in groups of 2 or 4 animals with $10^5$ tissue
culture infectious dose$_{50}$ (TCID$_{50}$) per ml of BPIV3 Ka, rHPIV3-F$_B$HN$_B$, rBPIV3-F$_H$HN$_H$,
rHPIV3-N$_B$ or rHPIV3. Nasopharyngeal swabs were collected daily on days 1 to 11 and
on day 13. Tracheal lavage samples were collected on days 2, 4, 6, 8, and 10 post-

5    infection. Individual samples were flash-frozen and stored at -70°C until all samples
were available for titration. Virus in the specimens was titered on LLC-MK2 cell
monolayers in 24 and 96 well plates as previously described (Durbin et al., 1999b, *supra*).
Sera collected from monkeys on days 0 and 28 was tested by HAI assay using HPIV3 JS
and BPIV3 Ka as antigens, as previously described (van Wyke Coelingh et al., 1988,

10   *supra*). On day 28 post inoculation, the monkeys were challenged intranasally and
intratracheally with $10^6$ TCID$_{50}$ per site of HPIV3 JS. Nasopharyngeal swab samples
were collected on days 3, 4, 5, 6, 7 and 8, and tracheal lavage samples on days 4, 6 and 8
post challenge. Samples were titered in a single assay as described above. Serum was
collected on day 28 post challenge.

15

**Recovery of rBPIV3 and BPIV3/HPIV3 chimeric viruses (rHPIV3-F$_B$HN$_B$ and
rBPIV3-F$_H$HN$_H$) from cDNA.**

A complete BPIV3 antigenomic cDNA, designated pBPIV(184), was
constructed to encode the consensus sequence of BPIV3 Ka. This BPIV3 antigenomic

20   cDNA was further modified by the introduction of unique *Sgr*AI and *Bsi*WI sites into the
downstream noncoding region of the M and HN genes, respectively (Fig. 8C). The same
restriction sites were introduced into the downstream noncoding region of the M and HN
genes of a previously described complete HPIV3 antigenomic cDNA, p3/7(131)2G
(Durbin et al., 1997a, *supra*). The F and HN glycoprotein genes of HPIV3 and BPIV3

25   were swapped by exchanging this *Sgr*AI-*Bsi*WI restriction fragment. A direct exchange
of entire genes was anticipated to be well-tolerated because of the high level of sequence
conservation between the cis-acting signals of BPIV3 and HPIV3. The HPIV3
antigenomic cDNA bearing the BPIV3 F and HN genes was designated pHPIV(215), and
the BPIV3 antigenomic cDNA bearing the HPIV3 F and HN genes was designated

30   pBPIV(215).

The antigenomic cDNAs pBPIV(184), pHPIV(215), pBPIV(215) and
p3/7(131)2G were separately transfected into HEp-2 cells along with the three BPIV3
support plasmids pTM(N), pTM(P) and pTM(L), and the cells were simultaneously

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infected with recombinant MVA expressing the T7 RNA polymerase. To confirm that the recovered viruses indeed were the expected rBPIV3, rHPIV3-$F_B HN_B$, rBPIV3-$F_H HN_H$ and rHPIV3 viruses, intracellular RNA or RNA from supernatant from each cloned virus was analyzed by RT-PCR using a primer pair that recognized identical sequences in

5    HPIV3 JS and BPIV3 Ka. The primer pair amplified a 4.8 kb fragment of DNA corresponding to the downstream end of the M gene, the F and HN genes, and the upstream end of the L gene (nts 4206-9035 in rBPIV3, nts 4224-9041 in rHPIV3, nts 4206-9017 in rBPIV3-$F_H HN_H$, and nts 4224-9059 in rHPIV3-$F_B HN_B$). The generation of each PCR product was dependent upon the inclusion of reverse transcriptase, indicating

10   that each was derived from viral RNA and not from contaminating cDNA (data not shown). The PCR products were then digested with *Eco* R1, which would be predicted to yield a different, unique restriction enzyme digest pattern for each of the four viruses (Figure 9). In each case, the predicted pattern was observed, confirming the identity of the backbone and the inserted F and HN genes. In addition, nucleotide sequencing was

15   performed on the RT-PCR products to confirm the presence of the introduced restriction sites and flanking sequences.

          The cytopathic effect (CPE) caused by rBPIV3-$F_H HN_H$ in LLC-MK2 cells was indistinguishable from that of HPIV3 JS (condensed, rounded-up cells and small syncytia) but different from BPIV3 (large multicellular syncytia), whereas the CPE

20   caused by rHPIV3-$F_B HN_B$ was identical to that caused by the BPIV3. This indicates that the cytopathology of the chimeric PIVs cosegregated with the parental origin of the F and HN genes.

**BPIV3/HPIV3 chimeric viruses replicate efficiently in cell culture.**

25         The growth kinetics of rHPIV3-$F_B HN_B$ and rBPIV3-$F_H HN_H$ were compared with that of their parental viruses by infecting LLC-MK2 monolayers at an MOI of 0.01 and monitoring the production of infectious virus. The kinetics and magnitude of replication of the two chimeric viruses were comparable to those of their HPIV3 or BPIV3 parental viruses (Figure 10). This suggested that BPIV3 and HPIV3

30   glycoproteins were compatible with the heterologous PIV3 internal proteins. This is an important property because it will be possible to efficiently prepare vaccine virus.

**The F and HN genes of the BPIV3/HPIV3 chimeric viruses are determinants of the host range restriction of replication of BPIV3 Ka in the respiratory tract of rhesus monkeys.**

rHPIV3-$F_B$HN$_B$ and rBPIV3-$F_H$HN$_H$ were evaluated for their ability to

5    replicate in the upper and lower respiratory tract of rhesus monkeys. In particular, the effects of introduction of the BPIV3 F and HN genes into HPIV3 on attenuation of replication in rhesus monkeys was demonstrated, as described above for the BPIV3 N protein (see also, Bailly et al., 2000, *supra*). In addition, the effects of introduction of the HPIV3 F and HN genes into BPIV3 on replication in rhesus monkeys was determined. If

10   the predominant attenuating mutations of BPIV3 were in genes other than the F and HN, then one would expect little overall effect of the HPIV3-BPIV3 glycoprotein exchange on replication of BPIV3 in rhesus monkeys.

Each chimeric virus was administered intranasally and intratracheally to rhesus monkeys at a dose of $10^5$ TCID$_{50}$ per site. The level of replication of the chimeric

15   viruses was compared to that of the rHPIV3 and BPIV3 parental viruses and to that of rHPIV3-$N_B$ (Table 3). Since the rHPIV3 parental virus replicated to a low to moderate level in the lower respiratory tract, meaningful comparisons between groups could only be made for replication in the upper respiratory tract. The level of replication of rHPIV3-$F_B$HN$_B$ was similar to that of its BPIV3 parent and substantially lower than that of its

20   HPIV3 parent (Table 3; Figure 11, panel A). This showed that the BPIV3 glycoprotein genes contained one or more major determinants of the host range attenuation phenotype of BPIV3 for rhesus monkeys. The magnitude and pattern of replication of rHPIV3-$F_B$HN$_B$ and rHPIV3-$N_B$ were very similar, indicating that each of the two bovine genetic elements, namely the N gene versus the F and HN genes, attenuate HPIV3 to a similar

25   extent.

Table 3. The F and HN glycoprotein genes of BPIV3 contribute to its restricted replication in the respiratory tract of rhesus monkeys.

| Immunizing virus[1] | Number of animals[2] | Mean peak virus titer[3] ($\log_{10}$ TCID$_{50}$/ml ± S.E.) [Duncan Grouping][4] | | Serum HAI antibody titer (mean recip. $\log_2$ ±S.E.) for HPIV3 on day 28[7] | Serum HAI antibody titer (mean recip. $\log_2$ ±S.E.) for BPIV3 on day 28[7] |
|---|---|---|---|---|---|
| | | NP swab[5] | Tracheal lavage[6] | | |
| rHPIV3 | 6 | 4.7±0.54 [A] | 2.4±0.37 [A] | 9.5±0.72 [A] | 6.8±1.03 [B] |
| rBPIV3-F$_H$HN$_H$ | 4 | 3.1±0.58 [B] | 1.6±0.05 [A] | 6.8±0.63 [BC] | 3.8±0.63 [C] |
| rHPIV3-N$_B$ | 6 | 3.0±0.60 [B] | 1.4±0.19 [A] | 8.2±0.48 [AB] | 6.5±0.62 [B] |
| rHPIV3-F$_B$HN$_B$ | 4 | 2.9±0.28 [B] | 2.0±0.24 [A] | 4.5±0.29 [D] | 9.5±0.65 [A] |
| BPIV3 Ka | 6 | 2.6±0.26 [B] | 1.6±0.10 [A] | 5.5±0.62 [CD] | 9.2±0.60 [A] |

[1] Monkeys were inoculated intranasally and intratracheally with $10^5$ TCID$_{50}$ of virus in a 1 ml inoculum at each site.

[2] The groups with 6 animals contain 4 animals each from a previous rhesus study (Bailly et al., 2000, supra).

[3] Mean of the peak virus titers for each animal in its group irrespective of sampling day. S.E. = standard error.

[4] Virus titrations were performed on LLC-MK2 cells at 32°C. The limit of detectability of virus titer was 10 TCID$_{50}$/ml. Mean viral titers were compared using a Duncan Multiple Range test (α= 0.05). Within each column, mean titers with different letters are statistically different. Titers indicated with two letters are not significantly different from those indicated with either letter.

[5] Nasopharyngeal swab samples were collected on days 1 to 11 and on day 13.

[6] Trachael lavage samples were collected on days 2, 4, 6, 8 and 10 post-infection.

[7] The titers on day 0 were <2.0. Day 28 was the day of challenge with wild type HPIV3.

** Two of the animals in the rHPIV3 group were infected with rHPIV3s, the virus containing two restriction enzyme recognition sites for the glycoprotein swap.

The rBPIV3-$F_H$HN$_H$ chimeric virus replicated significantly less well than rHPIV3 (Table 3), and it grouped with BPIV3 in a Duncan multiple range test. However, inspection of its pattern of replication in Figure 11B suggested that rBPIV3-$F_H$HN$_H$ replicated to a level intermediate between that of its HPIV3 and BPIV3 parents. The

5  interpretation that rBPIV3-$F_H$HN$_H$ replicates to a level intermediate between that of its parents is supported by Friedman's test of consistency of ranks (Sprent, P., "A Generalization Of The Sign Test," Applied Nonparametric Statistical Methods, pp. 123-126, Chapman and Hall, London, 1989, incorporated herein by reference), which indicated that the median titers of HPIV3, rBPIV3-$F_H$HN$_H$ , and BPIV3 between day 3

10  and day 8 post infection are significantly different (d.f.2,8; $p < 0.05$). The observation that the introduction of the HPIV3 F and HN proteins resulted in an increase in the replication of BPIV3 in rhesus monkeys indicates (i) that F and HN contain one or more determinants of host range restriction and (ii) that one or more genetic elements of BPIV3 that lie outside of the F and HN genes, e.g. the N protein, attenuate the virus for rhesus

15  monkeys. This confirms that the genetic basis for host range restriction can involve multiple genes.

**The chimeric BPIV3 bearing HPIV3 glycoprotein genes induces serum HAI antibody to HPIV3 and a high level of resistance to wt HPIV3 challenge.**

20  rBPIV3-$F_H$HN$_H$ has important features that make it a candidate live attenuated virus vaccine against HPIV3, including attenuating genes from BPIV3 and the antigenic specificity of HPIV3, i.e. the F and HN glycoproteins, which are the major protective antigens. Therefore, its immunogenicity and protective efficacy against challenge with HPIV3 were documented. Rhesus monkeys were immunized by infection

25  with BPIV3 Ka, rHPIV3-$F_B$HN$_B$, rBPIV3-$F_H$HN$_H$, rHPIV3-N$_B$, or rHPIV3. They were challenged 28 days later with HPIV3 JS wild type virus. Serum samples were taken prior to the initial infection on day 0 and prior to the challenge. BPIV3 and rHPIV3-$F_B$HN$_B$ induced serum HAI antibodies that reacted more efficiently with BPIV3 than HPIV3, whereas the converse was the case for HPIV3 and rBPIV3-$F_H$HN$_H$. Thus, the

30  origin of the glycoprotein genes in each virus determined whether the HAI antibody response was directed predominantly against HPIV3 or against BPIV3. The replication of challenge HPIV3 virus was significantly reduced in the upper and lower respiratory tract of previously immunized monkeys (Table 4). Although the level of protective efficacy against HPIV3 was not significantly different among the different viruses,

viruses bearing HPIV3 F and HN were consistently more protective in the upper
respiratory tract than were viruses bearing BPIV3 F and HN. This is in accordance with
the higher level of HPIV3-specific serum HAI antibodies induced by viruses bearing
HPIV3 F and HN.

5

Table 4. Immunization of rhesus monkeys with BPIV3/HPIV3 chimeric recombinant virus induces resistance to challenge with wild type HPIV3

| Immunizing virus[1] | Number of animals[2] | Mean peak virus titer[3] ($log_{10}$ TCID$_{50}$/ml ± S.E.) [Duncan Grouping][4] | | Serum HAI antibody titer (mean recip. $log_2$ ±S.E.) for HPIV3 on the day of challenge | Serum HAI antibody titer (mean recip. $log_2$ ±S.E.) for HPIV3 28 days after challenge |
|---|---|---|---|---|---|
| | | Nasopharyngeal swab[5] | Tracheal lavage[6] | | |
| none | 4 | 4.5±0.33 [A] | 4.5±0.19 [A] | <2 | 12.0±0.58 [A] |
| rHPIV3 | 6 | 2.3±0.14 [B] | 1.2±0.20 [B] | 9.5±0.72 [A] | 11.7±0.21 [A] |
| rBPIV3-F$_H$HN$_H$ | 4 | 2.5±0.25 [B] | 1.0±0.48 [B] | 6.8±0.63 [BC] | 10.5±0.29 [AB] |
| rHPIV3-N$_B$ | 6 | 2.3±0.41 [B] | 1.4±0.08 [B] | 8.2±0.48 [AB] | 11.5±0.22 [A] |
| rHPIV3-F$_B$HN$_B$ | 4 | 3.0±0.14 [B] | 1.0±0.0 [B] | 4.5±0.29 [D] | 9.5±0.87 [B] |
| BPIV3 Ka | 6 | 2.9±0.26 [B] | 1.3±0.20 [B] | 5.5±0.62 [CD] | 9.3±0.76 [B] |

[1] Each previously immunized monkey and non-immunized controls were challenged with $10^6$ TCID$_{50}$ of HPIV3 JS in a 1 ml inoculum at each site 28 days after immunization.

[2] The groups with 6 animals each contain 4 animals each from a previous rhesus study (Bailly et al., 2000, *supra*).

[3] Mean of the peak virus titers for each animal in its group irrespective of sampling day.

[4] Virus titrations were performed on LLC-MK2 cells. The limit of detectability of virus titer was 10 TCID$_{50}$/ml. Mean viral titers were compared using a Duncan Multiple Range test (α= 0.05). Within each column, mean titers with different letters are statistically different. Titers indicated with two letters are not significantly different from those indicated with either letter. The group of unimmunized animals were not included in the Duncan analysis at the day of challenge.

[5] Nasopharyngeal swab samples were collected on days 3 to 8 post challenge.

[6] Trachael lavage samples were collected on days 4, 6 and 8 post challenge.

** Two animals in the rHPIV3 group were infected with rHPIV3s.

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Based on the foregoing examples, the invention provides for importation
of BPIV genes into a virulent HPIV backbone and visa versa to yield novel, human-
bovine chimeric PIV vaccine candidates. In exemplary chimeric recombinants disclosed
in the present example, rBPIV3-$F_H HN_H$ and its rHPIV3-$F_B HN_B$ counterpart, replicated in

5      vitro as well as the respective parental viruses. It was also confirmed that the F and HN
exchange between the BPIV3 and HPIV3 is compatible since the considerably more
divergent HPIV1 F and HN proteins were highly functional in a HPIV3 background (Tao
et al., J. Virol. 72:2955-2961, 1998), which was evinced by the undiminished capacity of
the chimeric viruses for replication in vitro. rBPIV3-$F_H HN_H$ replicated in the upper

10     respiratory tract of rhesus monkeys to a level intermediate between that of its HPIV3 and
BPIV3 parents indicating that the BPIV3 F and HN genes make an independent
contribution to the overall attenuation of BPIV3 for primates. The overall attenuation of
BPIV3 virus thus is the sum of two or more genetic elements, one of which is the set of F
and HN genes and one of the others is indicated to be N.

15     Although BPIV3 itself is being evaluated as a vaccine virus for HPIV3
(Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; and Karron et al., J. Infect. Dis.
171:1107-1114, 1995), it is only 25% related antigenically to HPIV3 (Coelingh et al., J.
Infect. Dis. 157:655-662, 1988). Thus, the immunogenicity of BPIV3 against HPIV3 will
be improved if it is modified according to the present invention to express the protective

20     F and HN antigens of HPIV3. rBPIV3-$F_H HN_H$ represents such a virus, and, in the
present example, immunization of rhesus monkeys with rBPIV3-$F_H HN_H$ induced a higher
level of antibody to HPIV3 than did immunization with BPIV3. Furthermore, rBPIV3-
$F_H HN_H$ conferred a level of protection against replication of HPIV3 challenge in the
upper and lower respiratory tract that was statistically indistinguishable from that

25     conferred by a previous infection with rHPIV3. Similarly, rHPIV3-$N_B$, which is
attenuated by the BPIV3 N protein but possesses HPIV3 protective antigens, also induced
a high level of resistance to HPIV3 challenge. Despite replicating to similar levels in
rhesus monkeys, rHPIV3-$N_B$ induced higher levels of antibodies to HPIV3 than rBPIV3-
$F_H HN_H$.

30     rBPIV3-$F_H HN_H$ replicates to higher levels in rhesus monkeys than BPIV3,
although it is significantly attenuated compared to HPIV3. Since the level of replication
of BPIV3 in humans is low (Karron et al., J. Infect. Dis. 171:1107-1114, 1995), this
increase is expected to be well tolerated among vaccinees. Alternatively, additional
methods to attenuate human-bovine chimeric viruses of the invention are disclosed herein

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to ensure that the vaccine viruses replicate only to moderate levels, for example in human infants, to prevent unacceptable respiratory tract illness among vaccinees. Within other aspects of the invention, the slight increase in replication of rBPIV3-$F_H$HN$_H$ in primates offers an opportunity to use rBPIV3-$F_H$HN$_H$ as a vector for heterologous viral antigens

5    such as glycoproteins of other PIVs (e.g., HPIV1 and HPIV2), the RSV F and G glycoproteins, and the measles HA glycoprotein, which can be incorporated as added or substituted gene(s) or genome segment(s) into the attenuated HPIV3 vaccine candidate. In various alternative embodiments disclosed herein, the slight increase in replication of rBPIV3-$F_H$HN$_H$ in monkeys over that of BPIV3 can be offset by the addition of foreign

10   viral protective antigens, e.g., RSV glycoproteins, whose addition provides a selected level of attenuation. The data presented here further defined the basis for the host range restriction of BPIV3 for primates and identify rBPIV3-$F_H$HN$_H$ as a potential vaccine candidate against HPIV3 and as a vector for heterologous viral antigens.

Microorganism Deposit Information

15   The following materials have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, under the conditions of the Budapest Treaty and designated as follows:

| Plasmid | Accession No. | Deposit Date |
|---------|---------------|--------------|
| p3/7(131) | (ATCC 97990) | April 18, 1997 |
| p3/7(131)2G | (ATCC 97989) | April 18, 1997 |
| p218(131) | (ATCC 97991) | April 18, 1997 |

20

Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that

25   certain changes and modifications are comprehended by the disclosure and may be practiced without undue experimentation within the scope of the appended claims, which are presented by way of illustration not limitation.

## WHAT IS CLAIMED IS:

1.      An isolated infectious human-bovine chimeric parainfluenza virus (PIV) comprising a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), and a partial or complete PIV background genome or antigenome of a human PIV (HPIV) or bovine PIV (BPIV) combined with one or more

5     heterologous gene(s) or genome segment(s) of a different PIV to form a human-bovine chimeric PIV genome or antigenome.

2.      The chimeric PIV of claim 1, wherein said one or more heterologous gene(s) or genome segment(s) encodes one or more PIV N, P, C, D, V, M, F, HN and/or L protein(s) or fragment(s) thereof.

10     3.      The chimeric PIV of claim 1, wherein said one or more heterologous gene(s) or genome segment(s) encodes a complete open reading frame (ORF) of one or more PIV N, P, C, D, V, M, F, HN and/or L protein(s).

4.      The chimeric PIV of claim 1, wherein said one or more heterologous gene(s) or genome segment(s) includes a heterologous regulatory element

15     comprising an extragenic 3' leader or 5' trailer region, a gene-start signal, gene-end signal, RNA editing site, encapsidation signal, intergenic region, or 3' or 5' non-coding region.

5.      The chimeric PIV of claim 1, wherein said background genome or antigenome incorporates a heterologous genome segment integrated with the background

20     genome or antigenome to form a chimeric gene.

6.      The chimeric PIV of claim 5, wherein said chimeric genome or antigenome encodes a chimeric glycoprotein.

7.      The chimeric PIV of claim 1, wherein a heterologous gene or genome segment is substituted for a counterpart gene or genome segment in a partial PIV

25     background genome or antigenome.

8.      The chimeric PIV of claim 1, wherein a heterologous gene or genome segment is added adjacent to or within a noncoding region of the partial or complete PIV background genome or antigenome.

9.      The chimeric PIV of claim 1, wherein a heterologous gene or genome segment is added or substituted at a position corresponding to a wild-type gene order position of a counterpart gene or genome segment within the partial or complete PIV background genome or antigenome.

5      10.     The chimeric PIV of claim 1, wherein a heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of a counterpart gene or genome segment within the partial or complete PIV background genome or antigenome.

11.     The chimeric PIV of claim 1, wherein the chimeric genome or

10     antigenome comprises a partial or complete BPIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV.

12.     The chimeric PIV of claim 11, wherein one or more HPIV glycoprotein genes selected from HN and F, or one or more genome segments encoding a

15     cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope thereof, is/are substituted for one or more counterpart genes or genome segments within the BPIV background genome or antigenome.

13.     The chimeric PIV of claim 11, wherein one or more HPIV glycoprotein genes selected from HN and F is/are substituted to replace one or more

20     counterpart glycoprotein genes in the BPIV background genome or antigenome.

14.     The chimeric PIV of claim 13, wherein both HPIV glycoprotein genes HN and F are substituted to replace counterpart HN and F glycoprotein genes in the BPIV background genome or antigenome.

15.      The chimeric PIV of claim 13, which is rBPIV3-$F_H$HN$_H$.

25     16.     The chimeric PIV of claim 11, wherein the human-bovine chimeric PIV genome or antigenome encodes a chimeric glycoprotein having a HPIV glycoprotein ectodomain, antigenic determinant or immunogenic epitope.

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17.    The chimeric PIV of claim 16, wherein the heterologous genome segment encodes a glycoprotein ectodomain.

18.    The chimeric PIV of claim 11, wherein one or more HPIV glycoprotein genes HN and F, or a genome segment encoding a cytoplasmic domain,

5      transmembrane domain, ectodomain or immunogenic epitope thereof is added to or incorporated within a BPIV background genome or antigenome.

19.    The chimeric PIV of claim 11, wherein the chimeric genome or antigenome is further modified by addition or substitution of one or more additional heterologous gene(s) or genome segment(s) from a human PIV within the partial or

10     complete bovine background genome or antigenome to increase genetic stability or alter attenuation, reactogenicity or growth in culture of the chimeric virus.

20.    The chimeric PIV of claim 1, wherein the chimeric genome or antigenome comprises a partial or complete human PIV background genome or antigenome combined with one or more heterologous genes or genome segments from a

15     bovine PIV.

21.    The chimeric PIV of claim 20, wherein the heterologous gene or genome segment encodes a bovine PIV3 N protein.

22.    The chimeric PIV of claim 20, wherein a bovine PIV3 N open reading frame (ORF) is substituted for a human PIV3 N ORF in the chimeric genome or

20     antigenome.

23.    The chimeric PIV of claim 22, which is rHPIV3-N$_B$.

24.    The chimeric PIV of claim 1, wherein the genome or antigenome is further modified by introduction of one or more attenuating mutations identified in a biologically derived mutant PIV or other mutant nonsegmented negative stranded RNA

25     virus.

25.    The chimeric PIV of claim 24, wherein the genome or antigenome incorporates at least one and up to a full complement of attenuating mutations present within PIV3 JS cp45.

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26.     The chimeric PIV of claim 24, wherein the genome or antigenome incorporates at least one and up to a full complement of attenuating mutations specifying an amino acid substitution in the L protein at a position corresponding to Tyr$_{942}$, Leu$_{992}$, or Thr$_{1558}$ of JS; in the N protein at a position corresponding to residues Val$_{96}$ or Ser$_{389}$ of

5    JS, in the C protein at a position corresponding to Ile$_{96}$ of JS, in the M protein at a position corresponding to Pro$_{199}$ of JS in the F protein at a position corresponding to residues Ile$_{420}$ or Ala$_{450}$ of JS, in the HN protein at a position corresponding to residue Val$_{384}$ of JS, a nucleotide substitution a 3' leader sequence of the chimeric virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS cp45, and/or a mutation in an

10   N gene start sequence at a position corresponding to nucleotide 62 of JS cp45.

27.     The chimeric PIV of claim 24, wherein the genome or antigenome incorporates attenuating mutations from different biologically derived mutant PIVs.

28.     The chimeric PIV of claim 24, wherein the genome or antigenome incorporates an attenuating mutation at an amino acid position corresponding to an amino

15   acid position of an attenuating mutation identified in a heterologous, mutant negative stranded RNA virus.

29.     The chimeric PIV of claim 24, wherein the genome or antigenome includes at least one attenuating mutation stabilized by multiple nucleotide changes in a codon specifying the mutation.

20   30.     The chimeric PIV of claim 1, wherein the genome or antigenome comprises an additional nucleotide modification specifying a phenotypic change selected from a change in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host-range restriction, or a change in immunogenicity.

31.     The chimeric PIV of claim 30, wherein the additional nucleotide

25   modification alters one or more of the PIV N, P, C, D, V, M, F, HN and/or L genes and/or a 3' leader, 5' trailer RNA editing site, encapsidation signal, and/or an intergenic region.

32.     The chimeric PIV of claim 30, wherein one or more genes of the chimeric virus is deleted in whole or in part or expression of the genes is reduced or ablated by a mutation in an RNA editing site, by a frameshift mutation, by a mutation that

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alters an amino acid specified by an initiation codon, or by introduction of one or more

stop codons in an open reading frame (ORF) of the gene.

33.     The chimeric PIV of claim 30, wherein a modification is

introduced in the chimeric genome or antigenome comprising a partial or complete

5       deletion of one or more C, D and/or V ORF(s) or one or more nucleotide change(s) that

reduces or ablates expression of said one or more C, D and/or V ORF(s).

34.     The chimeric PIV of claim 30, wherein the chimeric genome or

antigenome is modified to encode a non-PIV molecule selected from a cytokine, a T-

lymphocyte helper epitope, a restriction site marker, or a protein of a microbial pathogen

10      capable of eliciting a protective immune response in a mammalian host.

35.     The chimeric PIV of claim 1, wherein the bovine-human chimeric

genome or antigenome comprises a partial or complete PIV vector genome or antigenome

combined with one or more heterologous genes or genome segments encoding one or

more antigenic determinants of one or more heterologous pathogens.

15      36.     The chimeric PIV of claim 35, wherein said one or more

heterologous pathogens is a heterologous PIV and said heterologous gene(s) or genome

segment(s) encode(s) one or more PIV N, P, C, D, V, M, F, HN and/or L protein(s) or

fragment(s) thereof.

37.     The chimeric PIV of claim 35, wherein the vector genome or

20      antigenome is a partial or complete HPIV genome or antigenome and the heterologous

gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more

heterologous PIV(s).

38.     The chimeric PIV of claim 37, wherein said one or more

heterologous PIV(s) is/are selected from HPIV1, HPIV2, or HPIV3.

25      39.     The chimeric PIV of claim 37, wherein the vector genome or

antigenome is a partial or complete HPIV genome or antigenome and the heterologous

gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more

heterologous HPIV(s).

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40.     The chimeric PIV of claim 39, wherein the vector genome or antigenome is a partial or complete HPIV3 genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more heterologous HPIV(s).

5          41.     The chimeric PIV of claim 40, wherein the chimeric genome or antigenome incorporates one or more gene(s) or genome segment(s) of a BPIV that specifies attenuation.

42.     The chimeric PIV of claim 35, wherein one or more HPIV1 or HPIV2 gene(s) or genome segment(s) encoding one or more HN and/or F glycoprotein(s)

10    or antigenic domain(s), fragment(s) or epitope(s) thereof is/are added to or incorporated within the partial or complete HPIV3 vector genome or antigenome.

43.     The chimeric PIV of claim 35, wherein both HPIV1 genes encoding HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or antigenome which is further modified by

15    addition or incorporation of one or more gene(s) or gene segment(s) encoding one or more antigenic determinant(s) of HPIV2 and one or more heterologous gene(s) or genome segment(s) of a BPIV that specifies attenuation.

44.     The chimeric PIV of claim 43, wherein a transcription unit comprising an open reading frame (ORF) of an HPIV2 HN or F gene is added to or

20    incorporated within the chimeric HPIV3-1 vector genome or antigenome.

45.     The chimeric PIV of claim 35, wherein the vector genome or antigenome is a partial or complete BPIV genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more HPIV(s).

25          46.     The chimeric PIV of claim 45, wherein said one or more antigenic determinant(s) is/are selected from HPIV1, HPIV2 or HPIV3 HN and F glycoproteins and antigenic domains, fragments and epitopes thereof.

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47.     The chimeric PIV of claim 45, wherein one or more gene(s) or genome segment(s) encoding one or more antigenic determinant(s) of HPIV2 is/are added to or substituted within the partial or complete BPIV vector genome or antigenome.

48.     The chimeric PIV of claim 45, wherein a plurality of heterologous genes or genome segments encoding antigenic determinants of multiple HPIVs are added to or incorporated within the partial or complete BPIV vector genome or antigenome.

49.     The chimeric PIV of claim 35, wherein the vector genome or antigenome is a partial or complete HPIV genome or antigenome and the heterologous pathogen is selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses.

50.     The chimeric PIV of claim 35, wherein the vector genome or antigenome is a partial or complete BPIV genome or antigenome and the heterologous pathogen is selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses.

51.     The chimeric PIV of claim 50, wherein said one or more heterologous antigenic determinant(s) is/are selected from measles virus HA and F proteins, subgroup A or subgroup B respiratory syncytial virus F, G, SH and M2 proteins, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, Flavivirus E and NS1 proteins, and alphavirus E protein, and antigenic domains, fragments and epitopes thereof.

52.     The chimeric PIV of claim 51, wherein the heterologous pathogen is measles virus and the heterologous antigenic determinant(s) is/are selected from the measles virus HA and F proteins and antigenic domains, fragments and epitopes thereof.

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53.     The chimeric PIV of claim 52, wherein a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to or incorporated within a HPIV3 vector genome or antigenome.

54.     The chimeric PIV of claim 51, which incorporates a gene or genome segment from respiratory syncytial virus (RSV).

55.     The chimeric PIV of claim 54, wherein the gene or genome segment encodes a RSV F and/or G glycoprotein or immunogenic domain(s) or epitope(s) thereof.

56.     The chimeric PIV of claim 1 which is a virus.

57.     The chimeric PIV of claim 1 which is a subviral particle.

58.     A method for stimulating the immune system of an individual to induce protection against PIV which comprises administering to the individual an immunologically sufficient amount of the chimeric PIV of claim 1 combined with a physiologically acceptable carrier.

59.     The method of claim 58, wherein the chimeric PIV is administered in a dose of $10^3$ to $10^7$ PFU.

60.     The method of claim 58, wherein the chimeric PIV is administered to the upper respiratory tract.

61.     The method of claim 58, wherein the chimeric PIV is administered by spray, droplet or aerosol.

62.     The method of claim 58, wherein the chimeric PIV and a second attenuated PIV are administered simultaneously as a mixture.

63.     An immunogenic composition to elicit an immune response against PIV comprising an immunogenically sufficient amount of the chimeric PIV of claim 1 in a physiologically acceptable carrier.

64.     The immunogenic composition of claim 63, formulated in a dose of $10^3$ to $10^7$ PFU.

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65.     The immunogenic composition of claim 63, formulated for administration to the upper respiratory tract by spray, droplet or aerosol.

66.     The immunogenic composition of claim 63, wherein the chimeric PIV elicits an immune response against one or more virus(es) selected from HPIV 1,

5     HPIV2 and HPIV3.

67.     The immunogenic composition of claim 66, wherein the chimeric PIV elicits an immune response against HPIV3 and another virus selected from HPIV 1, HPIV2 and HPIV3.

68.     An isolated polynucleotide molecule comprising a chimeric PIV

10     genome or antigenome which includes a partial or complete PIV background genome or antigenome of a human or bovine PIV combined with a heterologous gene or genome segment of a different PIV to form a human-bovine chimeric PIV genome or antigenome.

69.     The isolated polynucleotide of claim 68, wherein the chimeric genome or antigenome comprises a partial or complete BPIV background genome or

15     antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV.

70.     The isolated polynucleotide of claim 69, wherein one or more· HPIV glycoprotein genes selected from HN and F, or one or more genome segments encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic

20     epitope thereof, is/are substituted for one or more counterpart genes or genome segments within the BPIV background genome or antigenome.

71.     The isolated polynucleotide of claim 69, wherein one or more HPIV glycoprotein genes selected from HN and F is/are substituted to replace one or more counterpart glycoprotein genes in the BPIV background genome or antigenome.

25     72.     The isolated polynucleotide of claim 71, wherein both HPIV glycoprotein genes HN and F are substituted to replace counterpart HN and F glycoprotein genes in the BPIV background genome or antigenome.

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73.     The isolated polynucleotide of claim 71, which comprises a plasmid pBPIV(215).

74.     The isolated polynucleotide molecule of claim 68, wherein the chimeric genome or antigenome comprises a partial or complete human PIV background genome or antigenome combined with one or more heterologous genes or genome segments from a bovine PIV.

75.     The isolated polynucleotide of claim 74, wherein one or more BPIV glycoprotein genes selected from HN and F is/are substituted to replace one or more counterpart glycoprotein genes in the HPIV background genome or antigenome.

76.     The isolated polynucleotide of claim 75, wherein both BPIV glycoprotein genes HN and F are substituted to replace counterpart HN and F glycoprotein genes in the HPIV background genome or antigenome.

77.     The isolated polynucleotide of claim 76, which comprises a plasmid pHPIV(215).

78.     The isolated polynucleotide molecule of claim 74, wherein a bovine N gene is substituted to replace a counterpart N gene in a human PIV background genome or antigenome.

79.     The isolated polynucleotide molecule of claim 68, wherein the chimeric genome or antigenome is further modified by one or more attenuating mutations.

80.     The isolated polynucleotide molecule of claim 68, further comprising a nucleotide modification specifying a phenotypic change selected from a change in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host-range restriction, or a change in immunogenicity.

81.     A method for producing an infectious attenuated chimeric PIV particle from one or more isolated polynucleotide molecules encoding said PIV, comprising:

expressing in a cell or cell-free lysate an expression vector comprising an
isolated polynucleotide comprising a partial or complete PIV background genome or
antigenome of a human or bovine PIV combined with a heterologous gene or genome
segment of a different PIV to form a human-bovine chimeric PIV genome or antigenome,

5      and PIV N, P, and L proteins.

82.     The method of claim 81, wherein the chimeric PIV genome or
antigenome and the N, P, and L proteins are expressed by two or more different
expression vectors.

83.     An expression vector comprising an operably linked transcriptional
10     promoter, a polynucleotide sequence which includes a partial or complete PIV
background genome or antigenome of a human or bovine PIV combined with one or more
heterologous gene(s) or genome segment(s) of a different PIV to form a human-bovine
chimeric PIV genome or antigenome, and a transcriptional terminator.

Figure 1. Cloning f BPIV3 strain Ka or strain SF coding region into HPIV3 context

## A. Mutagenesis to create restriction sites at start and stop codons of N

HPIV3 N
pUC119JSN

CAAAAATGTTG                    GCAACTAATCGA

BPIV3 N
pBS-KaN or pBS-SFN

CAAAAATGTTG                    GCAACTAATCGA

HPIV3 N

TAACCATGGTGA                GCACTTAAGCAC
Ncol          pUC119JSN-Ncol/Afill        Afill

Ncol Afill digestion

BPIV3 N

TAACCATGGTGA                GCACTTAAGCAC
Ncol    pUC119KaN-Ncol/Afill        Afill
        or pUC119SFN-Ncol/Afill

Ncol Afill digestion

BPIV3 N coding

## B.

Ncol

Afill

Nco I                          Afl II

BPIV3 N

pUC119B/HKaN-Ncol/Afill or
pUC119B/HSFN-Ncol/Afill

## C. Mutagenesis to restore start and stop codon context

Legend

Ncol                          Afill
TAACCATGGTGA                  GCACTTAAGCAC

CAAAAATGTTGA                  GCAACTAGTCGA

BPIV3 N

Mlul        pUC119B/HKaN or          EcoRl
            pUC119B/HSFN

BPIV3 sequence
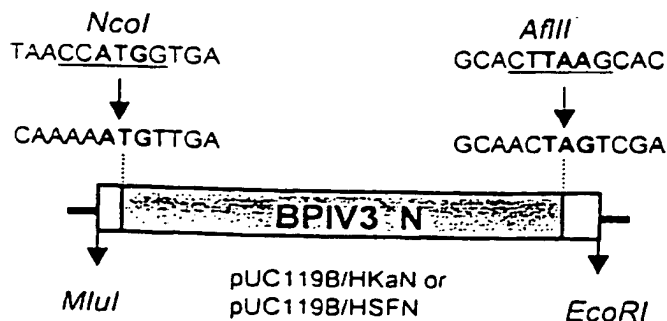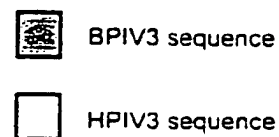
HPIV3 sequence

Figure 2. Cloning of BPIV3 N coding region into HPIV3 antigenomic cDNA
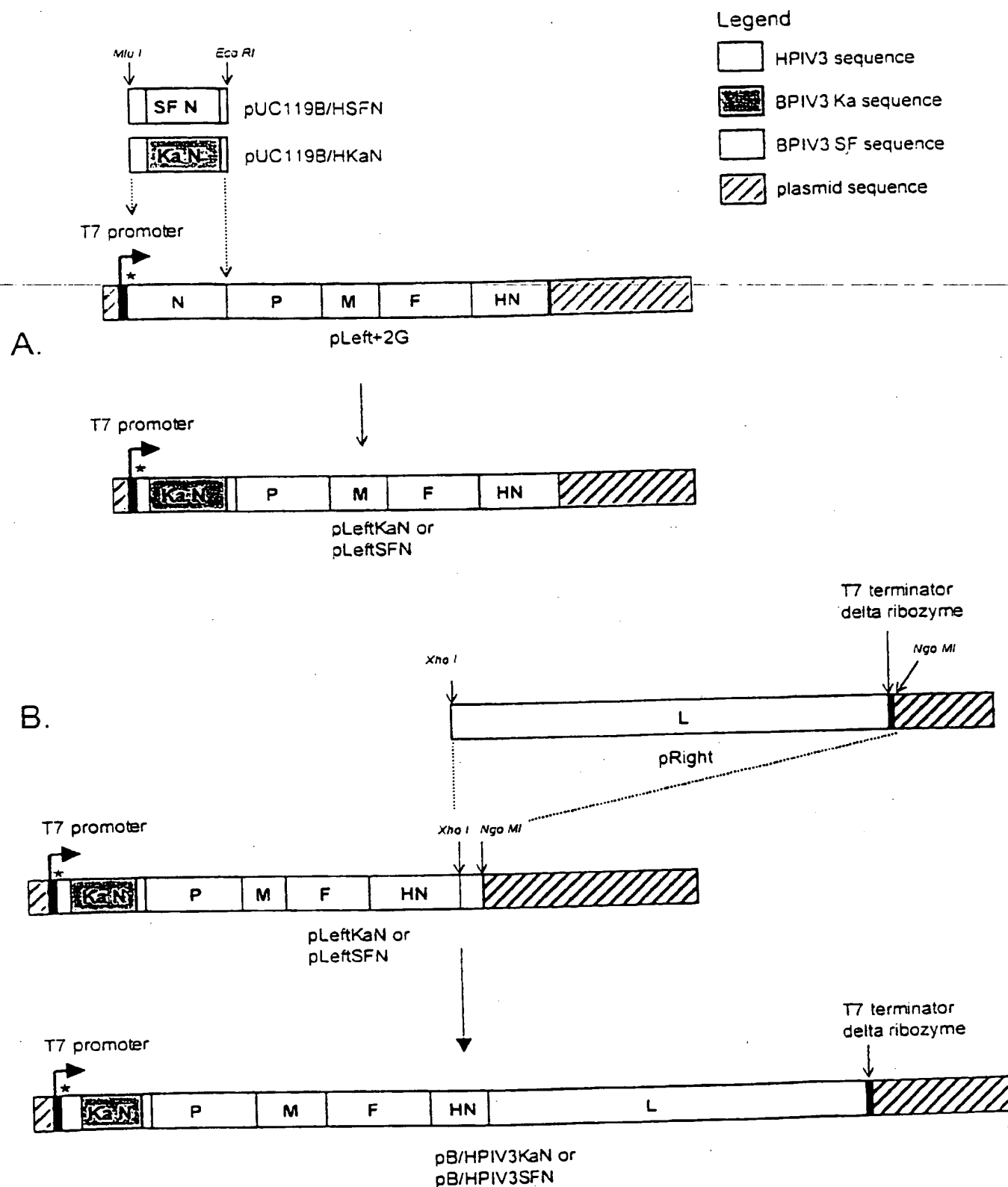
Figure 3. Nucleotide sequences of HPIV3, BPIV3 and chimeric viruses around the start (A) and stop (B) codons of the N gene

A.

rJS    GGAACTCTATAATTTCAAAAATGTTGAGCCTATTGATAC
cKa    GGAACTCTATAATTTCAAAAATGTTGAGTCTATTCGACAC
cSF    GGAACTCTATAATTTCAAAAATGTTGAGTCTATTCGACAC
Ka     GAAATCCTAAGACTGTAATCATGTTGAGTCTATTCGACAC
SF     GAAATCCTAAGACTGTAATCATGTTGAGTCTATTCGACAC

B.

rJS    TTAACGCATTTGGAAGCAACTAATCGAATCAACATTTTAA
cKa    TCAGTGCATTCGGAAGCAACTAGTCGAATCAACATTTTAA
cSF    TCAGTGCATTCGGAAGCAACTAGTCGAATCAACATTTTAA
Ka     TCAGTGCATTCGGAAGCAACTAGTCACAAAGAGATGACCA
SF     TCAGTGCATTCGGAAGCAACTAGTCACAAAGAGATGACCA

Figure 4. Confirmation of identity of potential BPIV3/HPIV3 chimeras by Taql digestion
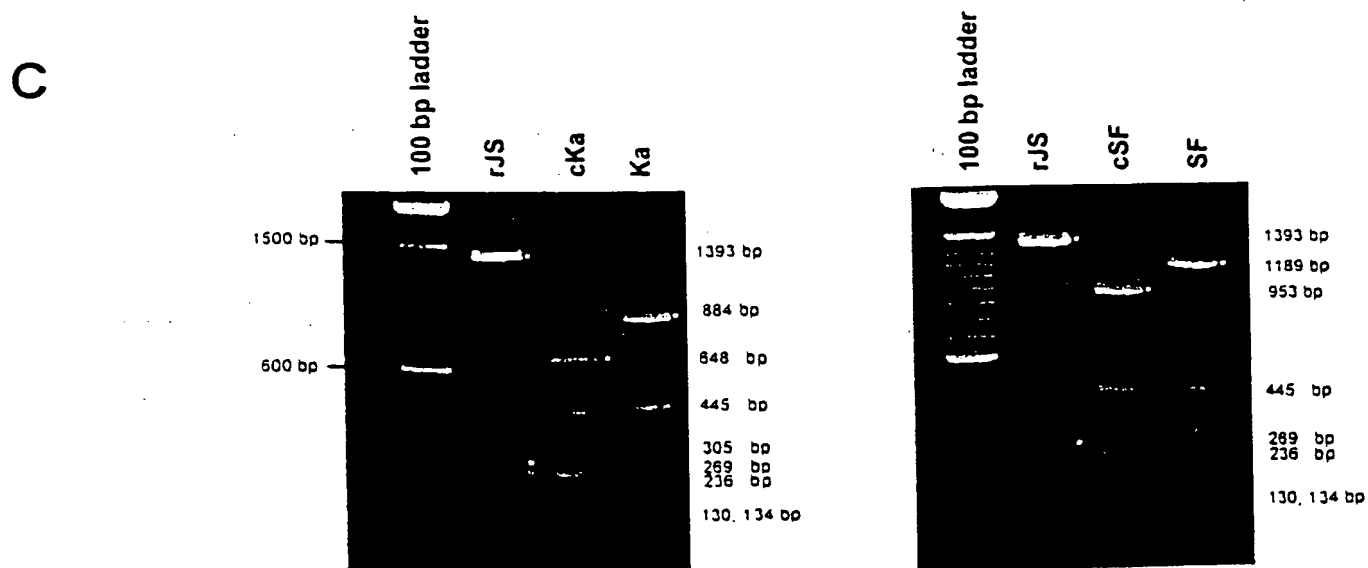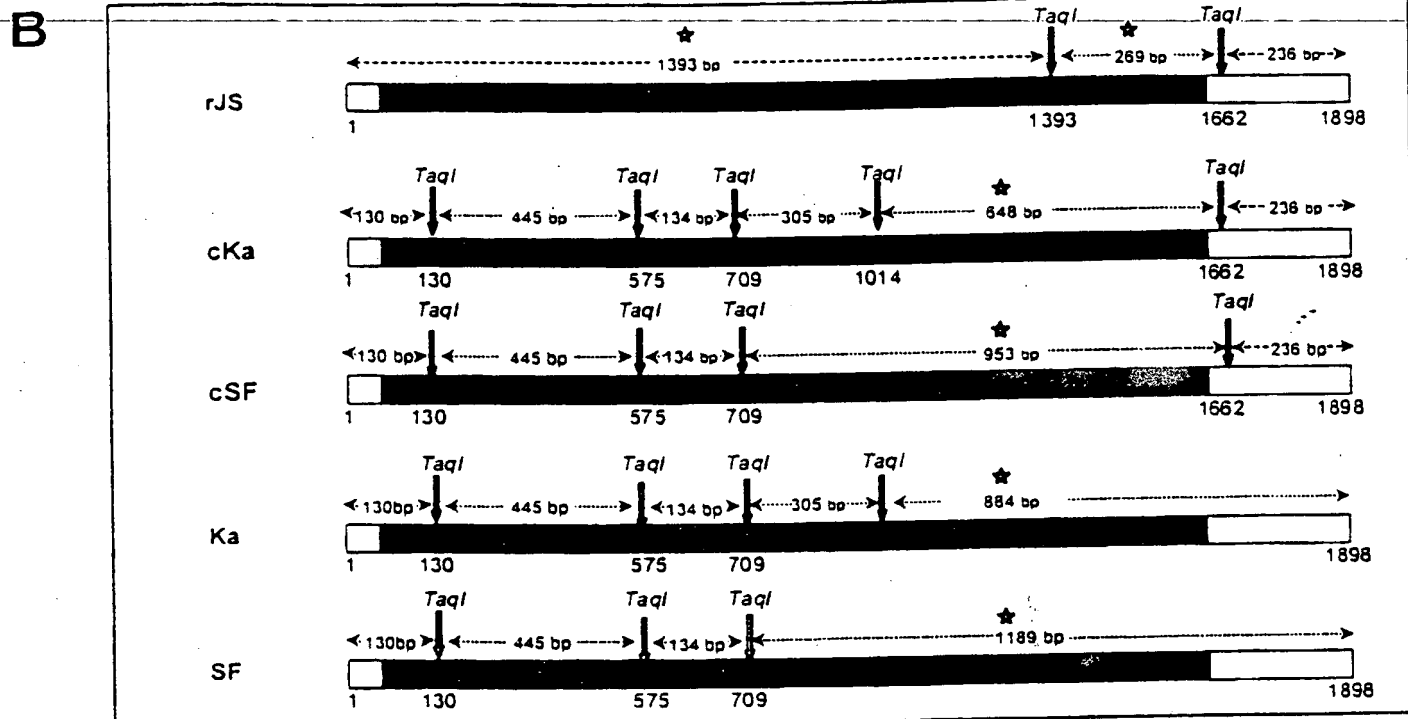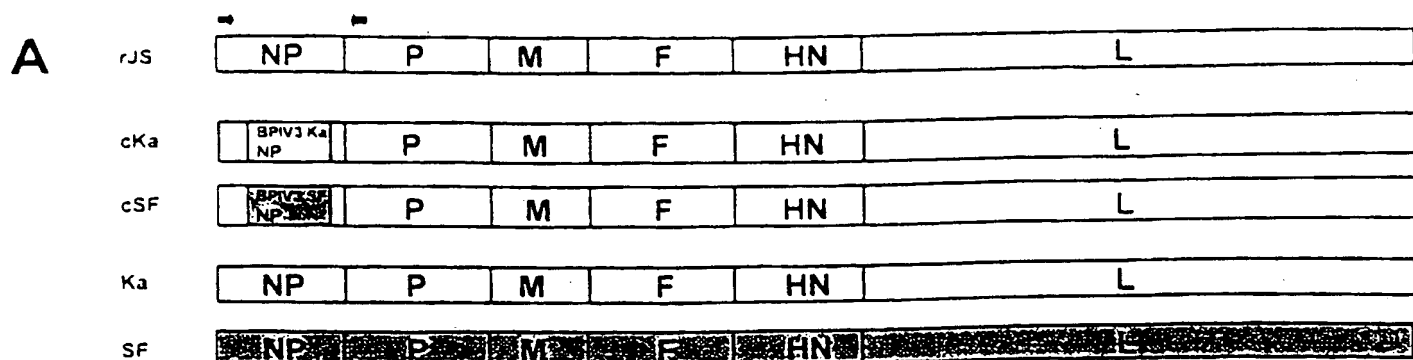
Figure 5. Multicycle growth curves in MDBK (A) or LLC-MK2 (B) cells
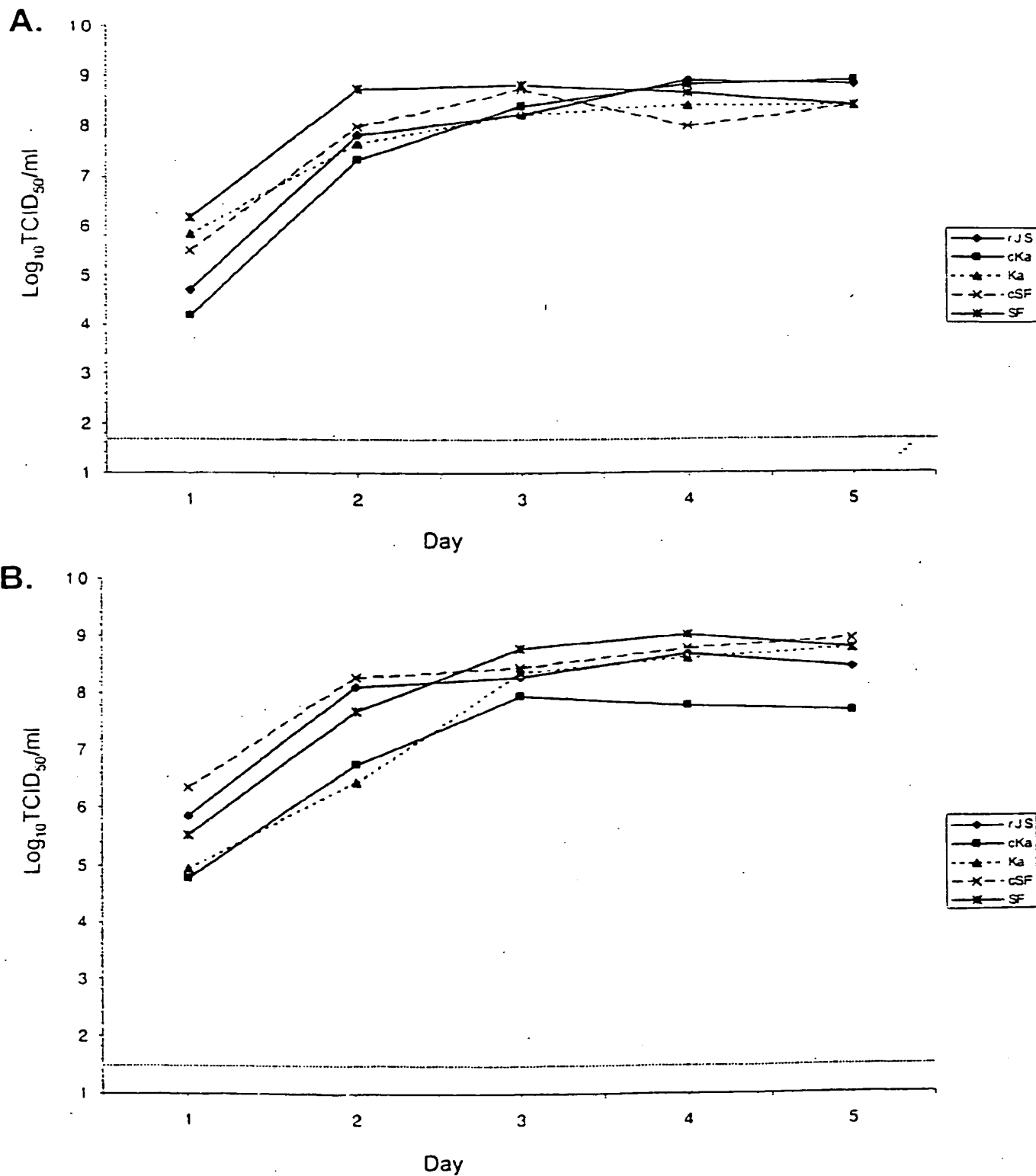
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ACCAAACAAG  AGAACAGACT  TGCTTGGGAA  TATTAATTCA  AATAAAAATT        50
AACTTAGGAT  TAAAGAACTT  TACCGAAAGG  TAAGGGGAAA  GAAATCCTAA       100
GACTGTAATC  ATGTTGAGTC  TATTCGACAC  ATTCAGTGCG  CGTAGGCAGG       150
AGAACATAAC  GAAATCAGCT  GGTGGGGCTG  TTATTCCCGG  GCAAAAAAAC       200
ACTGTGTCTA  TATTTGCTCT  TGGACCATCA  ATAACAGATG  ACAATGATAA       250
AATGACATTG  GCTCTTCTCT  TTTTGTCTCA  TTCTTTAGAC  AATGAAAAGC       300
AGCATGCGCA  AAGAGCTGGA  TTTTTAGTTT  CTCTGTTATC  AATGGCTTAT       350
GCCAACCCAG  AATTATATTT  AACATCAAAT  GGTAGTAATG  CAGATGTTAA       400
ATATGTTATC  TACATGATAG  AGAAAGACCC  AGGAAGACAG  AAATATGGTG       450
GGTTTGTCGT  CAAGACTAGA  GAGATGGTTT  ATGAAAAGAC  AACTGATTGG       500
ATGTTCGGGA  GTGATCTTGA  GTATGATCAA  GACAATATGT  TGCAAAATGG       550
TAGAAGCACT  TCTACAATCG  AGGATCTTGT  TCATACTTTT  GGATATCCAT       600
CGTGTCTTGG  AGCCCTTATA  ATCCAAGTTT  GGATAATACT  TGTTAAGGCT       650
ATAACCAGTA  TATCAGGATT  GAGGAAAGGA  TTCTTTACTC  GGTTAGAAGC       700
ATTTCGACAA  GATGGAACAG  TTAAATCCAG  TCTAGTGTTG  AGCGGTGATG       750
CAGTAGAACA  AATTGGATCA  ATTATGAGGT  CCCAACAGAG  CTTGGTAACA       800
CTCATGGTTG  AAACACTGAT  AACAATGAAC  ACAGGCAGGA  ATGATCTGAC       850
AACAATAGAA  AAGAATATAC  AGATTGTAGG  AAACTACATC  AGAGATGCAG       900
GTCTTGCTTC  ATTTTTCAAC  ACAATCAGAT  ATGGCATTGA  GACTAGAATG       950
GCAGCTCTAA  CTCTGTCTAC  CCTTAGACCG  GATATCAACA  GACTCAAGGC      1000
ACTGATCGAG  TTATATCTAT  CAAAGGGGCC  ACGTGCTCCT  TTTATATGCA      1050
TTTTGAGAGA  TCCCGTGCAT  GGTGAGTTTG  CACCAGGCAA  CTATCCTGCC      1100
CTCTGGAGTT  ATGCGATGGG  TGTAGCAGTT  GTACAAAACA  AGGCCATGCA      1150
ACAGTATGTA  ACAGGAAGGT  CTTATCTGGA  TATTGAAATG  TTCCAACTTG      1200
GTCAAGCAGT  GGCACGTGAT  GCCGAGTCGC  AGATGAGTTC  AATATTAGAG      1250
GATGAACTGG  GGGTCACACA  AGAAGCCAAG  CAAAGCTTGA  AGAAACACAT      1300
GAAGAACATC  AGCAGTTCAG  ATACAACCTT  TCATAAGCCT  ACAGGGGGAT      1350
CAGCCATAGA  AATGGCGATA  GATGAAGAAG  CAGGGCAGCC  TGAATCCAGA      1400
GGAGATCAGG  ATCAAGGAGA  TGAGCCTCGG  TCATCCATAG  TTCCTTATGC      1450
ATGGGCAGAC  GAAACCGGGA  ATGACAATCA  AACTGAATCA  ACTACAGAAA      1500
TTGACAGCAT  CAAAACTGAA  CAAAGAAACA  TCAGAGACAG  GCTGAACAAA      1550
AGACTCAACG  AGAAAAGGAA  ACAGAGTGAC  CCGAGATCAA  CTGACATCAC      1600
AAACAACACA  AATCAAACTG  AAATAGATGA  TTTGTTCAGT  GCATTCGGAA      1650
GCAACTAGTC  ACAAAGAGAT  GACCACTATC  ACCAGCAACA  AGTAAGAAAA      1700
ACTTAGGATT  AATGGAAATT  ATCCAATCCA  GAGACGGAAG  GACAAATCCA      1750
GAATCCAACC  ACAACTCAAT  CAACCAAAGA  TTCATGGAAG  ACAATGTTCA      1800
AAACAATCAA  ATCATGGATT  CTTGGGAAGA  GGGATCAGGA  GATAAATCAT      1850
CTGACATCTC  ATCGGCCCTC  GACATCATTG  AATTCATACT  CAGCACCGAC      1900
TCCCAAGAGA  ACACGGCAGA  CAGCAATGAA  ATCAACACAG  GAACCACAAG      1950
ACTTAGCACG  ACAATCTACC  AACCTGAATC  CAAAACAACA  GAAACAAGCA      2000
AGGAAAATAG  TGGACCAGCT  AACAAAAATC  GACAGTTTGG  GGCATCACAC      2050
GAACGTGCCA  CAGAGACAAA  AGATAGAAAT  GTTAATCAGG  AGACTGTACA      2100
GGGAGGATAT  AGGAGAGGAA  GCAGCCCAGA  TAGTAGAACT  GAGACTATGG      2150
TCACTCGAAG  AATCTCCAGA  AGCAGCCCAG  ATCCTAACAA  TGGAACCCAA      2200
ATCCAGGAAG  ATATTGATTA  CAATGAAGTT  GGAGAGATGG  ATAAGGACTC      2250
TACTAAGAGG  GAAATGCGAC  AATTTAAAGA  TGTTCCAGTC  AAGGTATCAG      2300
GAAGTGATGC  CATTCCTCCA  ACAAAACAAG  ATGGAGACGG  TGATGATGGA      2350
```

FIG. 6A

```
AGAGGCCTGG AATCTATCAG TACATTTGAT TCAGGATATA CCAGTATAGT    2400
GACTGCCGCA ACACTAGATG ACGAAGAAGA ACTCCTTATG AAGAACAACA    2450
GGCCAAGAAA GTATCAATCA ACACCCCAGA ACAGTGACAA GGGAATTAAA    2500
AAAGGGGTTG GAAGGCCAAA AGACACAGAC AAACAATCAT CAATATTGGA    2550
CTACGAACTC AACTTCAAAG GATCGAAGAA GAGCCAGAAA ATCCTCAAAG    2600
CCAGCACGAA TACAGGAGAA CCAACAAGAC CACAGAATGG ATCCCAGGGG    2650
AAGAGAATCA CATCCTGGAA CATCCTCAAC AGCGAGAGCG GCAATCGAAC    2700
AGAATCAACA AACCAAACCC ATCAGACATC AACCTCGGGA CAGAACCACA    2750
CAATGGGACC AAGCAGAACA ACCTCCGAAC CAAGGATCAA GACACAAAAG    2800
ACGGATGGAA AGGAAAGAGA GGACACAGAA GAGAGCACTC GATTTACAGA    2850
AAGGGCGATT ACATTATTAC AGAATCTTGG TGTAATCCAA TCTGCAGCAA    2900
AATTAGACCT ATACCAAGAC AAGAGAGTTG TGTGTGTGGC GAATGTCCTA    2950
AACAATGCAG ATACTGCATC AAAGATAGAC TTCCTAGCAG GTTTGATGAT    3000
AGGAGTGTCA ATGGATCATG ATACCAAATT AAATCAGATT CAGAACGAGA    3050
TATTAAGTTT GAAAACTGAT CTTAAAAAGA TGGATGAATC ACATAGAAGA    3100
CTAATTGAGA ATCAAAAAGA ACAATTATCA CTGATCACAT CATTAATCTC    3150
AAATCTTAAA ATTATGACAG AGAGGAGG GAAGAAGGAC CAACCAGAAC     3200
CTAGCGGGAG GACATCCATG ATCAAGACAA AAGCAAAGA AGAGAAAATA     3250
AAGAAAGTCA GGTTTGACCC TCTTATGGAA ACACAGGGCA TCGAGAAAAA    3300
CATCCCTGAC CTCTATAGAT CAATAGAGAA AACACCAGAA AACGACACAC    3350
AGATCAAATC AGAAATAAAC AGATTGAATG ATGAATCCAA TGCCACTAGA    3400.-
TTAGTACCTA GAAGAATAAG CAGTACAATG AGATCATTAA TAATAATCAT    3450
TAACAACAGC AATTTATCAT CAAAAGCAAA GCAATCATAC ATCAACGAAC    3500
TCAAGCTCTG CAAGAGTGAC GAGGAAGTGT CTGAGTTGAT GGACATGTTC    3550
AATGAGGATG TCAGCTCCCA GTAAACCGCC AACCAAGGGT CAACACCAAG    3600
AAAACCAATA GCACAAAACA GCCAATCAGA GACCACCCCA ATACACCAAA    3650
CCAATCAACA CATAACAAAG ATCTCCAGAT CATAGATGAT TAAGAAAAAC    3700
TTAGGATGAA AGGACTAATC AATCCTCCGA AACAATGAGC ATCACCAACT    3750
CCACAATCTA CACATTCCCA GAATCCTCTT TCTCCGAGAA TGGCAACATA    3800
GAGCCGTTAC CACTCAAGGT CAATGAACAG AGAAAGGCCA TACCTCATAT    3850
TAGGGTTGTC AAGATAGGAG ATCCGCCCAA ACATGGATCC AGATATCTGG    3900
ATGTCTTTTT ACTGGGCTTC TTTGAGATGG AAAGGTCAAA AGACAGGTAT    3950
GGGAGCATAA GTGATCTAGA TGATGATCCA AGTTACAAGG TTTGTGGCTC    4000
TGGATCATTG CCACTTGGGT TGGCTAGATA CACCGGAAAT GATCAGGAAC    4050
TCCTACAGGC TGCAACCAAG CTCGATATAG AAGTAAGAAG AACTGTAAAG    4100
GCTACGGAGA TGATAGTTTA CACTGTACAA AACATCAAAC TGAACTATA     4150
TCCATGGTCC AGTAGATTAA GAAAAGGGAT GTTATTTGAC GCTAATAAGG    4200
TTGCACTTGC TCCTCAATGT CTTCCACTAG ATAGAGGGAT AAAATTCAGG    4250
GTGATATTTG TGAACTGCAC AGCAATTGGA TCAATAACTC TATTCAAAAT    4300
CCCTAAGTCC ATGGCATTGT TATCATTGCC TAATACAATA TCAATAAATC    4350
TACAAGTACA TATCAAAACA GGAGTTCAGA CAGATTCCAA AGGAGTAGTT    4400
CAGATTCTAG ATGAAAAAGG TGAAAAATCA CTAAATTTCA TGGTTCATCT    4450
CGGGTTGATC AAAAGGAAGA TGGGCAGAAT GTACTCAGTT GAATATTGTA    4500
AGCAGAAGAT CGAGAAGATG AGATTATTAT TCTCATTGGG ATTAGTTGGA    4550
GGGATCAGCT TCCACGTCAA CGCAACTGGC TCTATATCAA AGACATTAGC    4600
AAGTCAATTA GCATTCAAAA GAGAAATCTG CTATCCCCTA ATGGATCTGA    4650
ATCCACACTT AAATTCAGTT ATATGGGCAT CATCAGTTGA AATTACAAGG    4700
```

FIG. 6B

```
GTAGATGCAG TTCTCCAGCC TTCATTACCT GGCGAATTCA GATACTACCC    4750
AAACATCATA GCAAAAGGGG TCGGGAAAAT CAGACAGTAA AATCAACAAC    4800
CCTGATATCC AACATTGCAA ATCAGGCTAC CCACAGGAGA AAAATCAAAA    4850
ACTTAGGATC AAAGGGATCA CCACGAACCC CGGAAAACAG CCAAACAAAC    4900
CAACACACAA ATCACAGACA AAAAGGAGAA GGCACTGCAA AGACCGAGAA    4950
AAAACAGAAC GCACACAACC AAGCAGAGAA AAGCCAAAGC CCGCCATTCA    5000
CAAACACACC AACAATCCTG CAAACAAGCA CCAAACAGA GGTCAAAAGA     5050
CAAAGAGCAC CAGATATGAC CATCACAACC ACAATCATAG CCATATTACT    5100
AATACCCCCA TCATTTTGTC AAATAGACAT AACAAAACTG CAACGTGTAG    5150
GTGTGTTAGT CAACAATCCT AAAGGCATGA AGATTTCACA AAATTTCGAA    5200
ACGAGATACC TGATATTAAG TTTGATACCC AAAATAGAGA ATTCACACTC    5250
ATGTGGGGAT CAACAGATAA ACCAATACAA GAAGTTATTG GATAGATTGA    5300
TAATTCCTCT ATATGATGGA TTAAAATTAC AAAAAGATGT AATAGTAGTA    5350
AGTCATGAAA CCCACAACAA TACTAATCTT AGGACAAAAC GATTCTTTGG    5400
AGAGATAATT GGGACAATTG CGATAGGGAT AGCCACTTCA GCACAAATCA    5450
CCGCAGCAGT CGCTCTTGTC GAAGCTAAAC AGGCAAAGTC AGACATAGAA    5500
AAACTCAAAG AGGCTATAAG AGACACAAAC AAGGCAGTAC AATCGATTCA    5550
AAGTTCTGTA GGTAACCTAA TTGTTGCAGT TAAATCAGTT CAAGACTATG    5600
TCAACAATGA AATTATACCT TCAATCACAA GATTAGGCTG TGAAGCAGCA    5650
GGGTTACAAT TGGGAATTGC ATTGACACAA CATTACTCAG AATTAACAAA    5700
TATATTTGGT GATAATATAG GAACACTGAA AGAAAAAGGG ATAAAATTAC    5750
AAGGGATAGC ATCATTATAT CACACAAACA TAACGGAAAT ATTTACTACT    5800
TCAACAGTTG ACCAATATGA TATTTATGAC CTATTATTCA CTGAGTCAAT    5850
CAAGATGAGA GTGATAGATG TTGATTTGAG TGATTACTCA ATTACTCTTC    5900
AAGTTAGACT TCCTTTATTA ACTAAACTAT CAAATACTCA AATTTATAAA    5950
GTAGATTCTA TATCATACAA CATCCAGGGC AAAGAGTGGT ATATTCCTCT    6000
TCCCAATCAC ATCATGACAA AAGGGGCTTT TCTAGGTGGT GCTGATATTA    6050
AAGAATGCAT AGAGGCATTC AGCAGTTATA TATGTCCTTC TGATCCAGGT    6100
TACATATTAA ATCACGAGAT AGAGAATTGT TTATCAGGGA ACATAACACA    6150
GTGTCCTAAG ACTGTTGTTA CATCAGATGT GGTACCACGA TACGCGTTTG    6200
TGAATGGTGG ATTAATTGCA AACTGCATAA CAACTACATG TACATGCAAT    6250
GGAATTGACA ATAGAATTAA TCAATCACCT GATCAAGGAA TTAAGATCAT    6300
AACACATAAA GAATGCCAGG TAATAGGTAT AAACGGAATG TTATTCAATA    6350
CTAATAGAGA AGGGACATTA GCAACTTATA CATTTGATGA CATCATATTA    6400
AATAACTCTG TTGCACTTAA TCCAATTGAT ATATCTATGG AACTCAACAA    6450
GGCAAAACTA GAATTAGAAG AATCGAAGGA ATGGATAAAG AAATCAAATC    6500
AAAAGTTAGA TTCCGTTGGA AGTTGGTATC AATCTAGTGC AACAATCACC    6550
ATAATCATAG TGATGATAAT AATTCTAGTT ATAATCAATA TAACAATTAT    6600
TGTAGTCATA ATCAAATTCC ATAGAATTCA GGGGAAAGAT CAAAACGACA    6650
AAAACAGTGA GCCGTATATA CTGACAAATA GACAATAAGA CTATACACGA    6700
TCAAATATAA AAAGTACAAA AAACTTAGGA ACAAAGTTGT TCAACACAGC    6750
AGCACCGAAT AGACCAAAAG CAGCGCAGA GGCGACACCA AACTCAAAAA     6800
TGGAATATTG GAAACACACA AACAGCATAA ATAACACCAA CAATGAAACC    6850
GAAACAGCCA GAGGCAAACA TAGTAGCAAG GTTACAAATA TCATAATGTA    6900
CACCTTCTGG ACAATAACAT TAACAATATT ATCAGTCATT TTTATAATGA    6950
TATTGACAAA CTTAATTCAA GAGAACAATC ATAATAAATT AATGTTGCAG    7000
GAAATAAGAA AAGAATTCGC GGCAATAGAC ACCAAGATTC AGAGGACTTC    7050
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FIG. 6C

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GGATGACATT GGAACCTCAA TACAGTCAGG AATAAATACA AGACTTCTCA      7100
CAATTCAGAG TCATGTTCAA AACTATATCC CACTATCATT AACACAACAA      7150
ATGTCAGATC TCAGAAAATT TATCAATGAT CTAACAAATA AAAGAGAACA      7200
TCAAGAAGTG CCAATACAGA GAATGACTCA TGATAGAGGT ATAGAACCCC      7250
TAAATCCAAA CAAGTTCTGG AGGTGTACAT CTGGTAACCC ATCTCTAACA      7300
AGTAGTCCTA AGATAAGGTT AATACCAGGA CCAGGTTTAT TAGCAACATC      7350
TACTACAGTA AATGGCTGTA TTAGAATTCC ATCGTTAGTA ATCAATCATC      7400
TAATCTATGC TTACACCTCT AATCTTATTA CCCAGGGCTG TCAAGATATA      7450
GGGAAATCTT ACCAAGTACT ACAAATAGGG ATAATTACTA TAAATTCGGA      7500
CCTAGTACCT GATTAAACC CCAGAGTCAC ACATACATTT AATATTGATG       7550
ATAATAGAAG ATCTTGCTCT CTGGCACTAT TGAATACAGA TGTTTATCAG      7600
TTATGCTCAA CACCAAAAGT TGATGAAAGA TCCGATTATG CATCAACAGG      7650
TATTGAGGAT ATTGTACTTG ACATTGTCAC TAATAATGGA TTAATTATAA      7700
CAACAAGGTT TACAAATAAT AATATAACTT TTGATAAACC GTATGCAGCA      7750
TTGTATCCAT CAGTGGGACC AGGAATCTAT TATAAGGATA AAGTTATATT      7800
TCTCGGATAT GGAGGTCTAG AGCATGAAGA AAACGGAGAC GTAATATGTA      7850
ATACAACTGG TTGTCCTGGC AAAACACAGA GAGACTGTAA TCAGGCTTCT      7900
TATAGCCCAT GGTTCTCAAA TAGGAGAATG GTAAACTCTA TTATTGTTGT      7950
TGATAAAGGC ATAGATGCAA CTTTTAGCTT GAGGGTGTGG ACTATTCCAA      8000
TGAGCCAAAA TTATTGGGGA TCAGAAGGAA GATTACTTTT ATTAGGTGAC      8050
AGAATATACA TATATACTAG ATCCACAAGT TGGCACAGTA AATTACAGTT      8100
AGGGGTAATT GATATTTCTG ATTATACTAA TATAAGAATA AATTGGACTT      8150
GGCATAATGT ACTATCACGG CCAGGGAATG ATGAATGTCC ATGGGGTCAT      8200
TCATGCCCAG ACGGATGTAT AACAGGAGTT TACACTGATG CATATCCGCT      8250
AAACCCATCG GGGAGTGTTG TATCATCAGT AATTCTTGAT TCACAAAGT       8300
CTAGAGAAAA CCCAATCATT ACTTACTCAA CAGCTACAAA TAGAATAAAT      8350
GAATTAGCTA TATATAACAG AACACTTCCA GCTGCATATA CAACAACAAA      8400
TTGTATCACA CATTATGATA AAGGGTATTG TTTTCATATA GTAGAAATAA      8450
ATCACAGAAG TTTGAATACG TTTCAACCTA TGTTATTCAA AACAGAAGTT      8500
CCAAAAAACT GCAGCTAAAT TGATCATCGC ATATCGGATG CAAGATGACA      8550
TTAAAAGAGA CCACCAGACA GACAACACAG GAGACGATGC AAGATATAAA      8600
GAAATAATAA AAAACTTAGG AGAAAAGTGT GCAAGAAAAA TGGACACCGA      8650
GTCCCACAGC GGCACAACAT CTGACATTCT GTACCCTGAA TGTCACCTCA      8700
ATTCTCCTAT AGTTAAAGGA AAGATAGCAC AACTGCATAC AATAATGAGT      8750
TTGCCTCAGC CCTACGATAT GGATGATGAT TCAATACTGA TTATTACTAG      8800
ACAAAAAATT AAACTCAATA AATTAGATAA AAGACAACGG TCAATTAGGA      8850
AATTAAGATC AGTCTTAATG GAAAGAGTAA GTGATCTAGG TAAATATACC      8900
TTTATCAGAT ATCCAGAGAT GTCTAGTGAA ATGTTCCAAT TATGTATACC      8950
CGGAATTAAT AATAAAATAA ATGAATTGCT AAGTAAAGCA AGTAAAACAT      9000
ATAATCAAAT GACTGATGGA TTAAGAGATC TATGGGTTAC TATACTATCG      9050
AAGTTAGCAT CGAAAAATGA TGGAAGTAAT TATGATATCA ATGAAGATAT      9100
TAGCAATATA TCAAATGTTC ACATGACTTA TCAATCAGAC AAATGGTATA      9150
ATCCATTCAA GACATGGTTT ACTATTAAGT ATGACATGAG AAGATTACAA      9200
AAAGCCAAAA ATGAGATTAC ATTCAATAGG CATAAAGATT ATAATCTATT      9250
AGAAGACCAA AAGAATATAT TGCTGATACA TCCAGAACTC GTCTTAATAT      9300
TAGATAAACA AAATTACAAT GGGTATATAA TGACTCCTGA ATTGGTACTA      9350
ATGTATTGTG ATGTAGTTGA AGGGAGGTGG AATATAAGTT CATGTGCAAA      9400
```

FIG. 6D

```
ATTGGATCCT AAGTTACAAT CAATGTATTA TAAGGGTAAC AATTTATGGG       9450
AAATAATAGA TGGACTATTC TCGACCTTAG GAGAAAGAAC ATTTGACATA       9500
ATATCACTAT TAGAACCACT TGCATTATCG CTCATTCAAA CTTATGACCC       9550
GGTTAAACAG CTCAGGGGGG CTTTTTTAAA TCACGTGTTA TCAGAAATGG       9600
AATTAATATT TGCAGCTGAG TGTACAACAG AGGAAATACC TAATGTGGAT       9650
TATATAGATA AAATTTTAGA TGTGTTCAAA GAATCAACAA TAGATGAAAT       9700
AGCAGAAATT TTCTCTTTCT TCCGAACTTT TGGACACCCT CCATTAGAGG       9750
CGAGTATAGC AGCAGAGAAA GTTAGAAAGT ATATGTATAC TGAGAAATGC       9800
TTGAAATTTG ATACTATCAA TAAATGTCAT GCTATTTTTT GTACAATAAT       9850
TATAAATGGA TATAGAGAAA GACATGGTGG TCAATGGCCT CCAGTTACAT       9900
TACCTGTCCA TGCACATGAA TTTATCATAA ATGCATACGG ATCAAATTCT       9950
GCCATATCAT ATGAGAATGC TGTAGATTAT TATAAGAGCT TCATAGGAAT      10000
AAAATTTGAC AAGTTTATAG AGCCTCAATT GGATGAAGAC TTAACTATTT      10050
ATATGAAAGA TAAAGCATTA TCCCCAAAGA AATCAAACTG GGACACAGTC      10100
TATCCAGCTT CAAACCTGTT ATACCGCACT AATGTGTCTC ATGATTCACG      10150
AAGATTGGTT GAAGTATTTA TAGCAGATAG TAAATTTGAT CCCCACCAAG      10200
TATTAGATTA CGTAGAATCA GGATATTGGC TGGATGATCC TGAATTTAAT      10250
ATCTCATATA GTTTAAAAGA GAAAGAAATA AAACAAGAAG GTAGACTTTT      10300
TGCAAAAATG ACATACAAGA TGAGGGCTAC ACAAGTATTA TCAGAAACAT      10350
TATTGGCGAA TAATATAGGG AAATTCTTCC AAGAGAATGG GATGGTTAAA      10400
GGAGAAATTG AATTACTCAA GAGACTAACA ACAATATCTA TGTCTGGAGT      10450
TCCGCGGTAT AATGAGGTAT ACAATAATTC AAAAAGTCAC ACAGAAGAAC      10500
TTCAAGCTTA TAATGCAATT AGCAGTTCCA ATTTATCTTC TAATCAGAAG      10550
TCAAAGAAGT TTGAATTTAA ATCTACAGAT ATATACAATG ATGGATACGA      10600
AACCGTAAGC TGCTTCTTAA CGACAGATCT TAAAAAATAT TGTTTAAATT      10650
GGAGGTATGA ATCAACAGCT TTATTCGGTG ATACTTGTAA TCAGATATTT      10700
GGGTTAAAGG AATTATTTAA TTGGCTGCAC CCTCGCCTTG AAAAGAGTAC      10750
AATATATGTT GGAGATCCTT ATTGCCCGCC ATCAGATATT GAACATTTAC      10800
CACTTGATGA CCATCCTGAT TCAGGATTTT ATGTTCATAA TCCTAAAGGA      10850
GGAATAGAAG GGTTTTGCCA AAAGTTATGG ACACTCATAT CTATCAGTGC      10900
AATACATTTA GCAGCTGTCA AAATCGGTGT AAGAGTTACT GCAATGGTTC      10950
AAGGGGATAA TCAAGCCATA GCTGTTACCA CAAGAGTACC TAATAATTAT      11000
GATTATAAAG TTAAGAAAGA GATTGTTTAT AAAGATGTGG TAAGATTTTT      11050
TGATTCCTTG AGAGAGGTGA TGGATGATCT GGGTCATGAG CTCAAACTAA      11100
ATGAAACTAT AATAAGTAGT AAAATGTTTA TATATAGCAA AAGGATATAC      11150
TATGACGGAA GAATCCTTCC TCAGGCATTA AAAGCATTGT CTAGATGTGT      11200
TTTTTGGTCT GAAACAATCA TAGATGAGAC AAGATCAGCA TCCTCAAATC      11250
TGGCTACATC GTTTGCAAAG GCCATTGAGA ATGGCTACTC ACCTGTATTG      11300
GGATATGTAT GCTCAATCTT CAAAAATATC CAACAGTTGT ATATAGCGCT      11350
TGGAATGAAT ATAAACCCAA CTATAACCCA AAATATTAAA GATCAATATT      11400
TCAGGAATAT TCATTGGATG CAATATGCCT CCTTAATCCC TGCTAGTGTC      11450
GGAGGATTTA ATTATATGGC CATGTCAAGG TGTTTTGTCA GAAACATTGG      11500
AGATCCTACA GTCGCTGCGT TAGCCGATAT TAAAAGATTT ATAAAAGCAA      11550
ATTTGTTAGA TCGAGGTGTC CTTTACAGAA TTATGAATCA AGAACCAGGC      11600
GAGTCTTCTT TTTTAGACTG GGCCTCAGAT CCCTATTCAT GTAACTTACC      11650
ACAATCTCAA AATATAACCA CCATGATAAA GAATATAACT GCAAGAAATG      11700
TACTACAGGA CTCACCAAAC CCATTACTAT CTGGATTATT TACAAGTACA      11750
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FIG. 6E

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ATGATAGAAG AGGATGAGGA ATTAGCTGAG TTCCTAATGG ACAGGAGAAT    11800
AATCCTCCCA AGAGTTGCAC ATGACATTTT AGATAATTCT CTTACTGGAA    11850
TTAGGAATGC TATAGCTGGT ATGTTGGATA CAACAAAATC ACTAATTCGA    11900
GTAGGGATAA GCAGAGGAGG ATTAACCTAT AACTTATTAA GAAAGATAAG    11950
CAACTATGAT CTTGTACAAT ATGAGACACT TAGTAAAACT TTAAGACTAA    12000
TAGTCAGTGA CAAGATTAAG TATGAAGATA TGTGCTCAGT AGACCTAGCC    12050
ATATCATTAA GACAAAAAAT GTGGATGCAT TTATCAGGAG GAAGAATGAT    12100
AAATGGACTT GAAACTCCAG ATCCTTTAGA GTTACTGTCT GGAGTAATAA    12150
TAACAGGATC TGAACATTGT AGGATATGTT ATTCAACTGA AGGTGAAAGC    12200
CCATATACAT GGATGTATTT ACCAGGCAAT CTTAATATAG GATCAGCTGA    12250
GACAGGAATA GCATCATTAA GGGTCCCTTA CTTTGGATCA GTTACAGATG    12300
AGAGATCTGA AGCACAATTA GGGTATATCA AAAATCTAAG CAAACCAGCT    12350
AAGGCTGCTA TAAGAATAGC AATGATATAT ACTTGGGCAT TTGGGAATGA    12400
CGAAATATCT TGGATGGAAG CATCACAGAT TGCACAAACA CGTGCAAACT    12450
TTACATTGGA TAGCTTAAAG ATTTTGACAC CAGTGACAAC ATCAACAAAT    12500
CTATCACACA GGTTAAAAGA TACTGCTACT CAGATGAAAT TTTCTAGTAC    12550
ATCACTTATT AGAGTAAGCA GGTTCATCAC AATATCTAAT GATAATATGT    12600
CTATTAAAGA AGCAAATGAA ACTAAAGATA CAAATCTTAT TTATCAACAG    12650
GTAATGTTAA CAGGATTAAG TGTATTTGAA TATCTATTTA GGTTAGAGGA    12700
GAGTACAGGA CATAACCCTA TGGTCATGCA TCTACATATA GAGGATGGAT    12750
GTTGTATAAA AGAGAGTTAC AATGATGAGC ATATCAATCC GGAGTCTACA    12800
TTAGAGTTAA TCAAATACCC TGAGAGTAAT GAATTTATAT ATGATAAGGA    12850
CCCTTTAAAG GATATAGATC TATCAAAATT AATGGTTATA AGAGATCATT    12900
CTTATACAAT TGACATGAAT TACTGGGATG ACACAGATAT TGTACATGCA    12950
ATATCAATAT GTACTGCAGT TACAATAGCA GATACAATGT CGCAGCTAGA    13000
TCGGGATAAT CTTAAGGAGC TGGTTGTGAT TGCAAATGAT GATGATATTA    13050
ACAGTCTGAT AACTGAATTT CTGACCCTAG ATATACTAGT GTTTCTCAAA    13100
ACATTTGGAG GGTTACTCGT GAATCAATTT GCATATACCC TTTATGGATT    13150
GAAAATAGAA GGAAGGGATC CCATTTGGGA TTATATAATG AGAACATTAA    13200
AAGACACCTC ACATTCAGTA CTTAAAGTAT TATCTAATGC ACTATCTCAT    13250
CCAAAAGTGT TTAAGAGATT TTGGGATTGT GGAGTTTTGA ATCCTATTTA    13300
TGGTCCTAAT ACTGCTAGTC AAGATCAAGT TAAGCTTGCT CTCTCGATTT    13350
GCGAGTACTC CTTGGATCTA TTTATGAGAG AATGGTTGAA TGGAGCATCA    13400
CTTGAGATCT ATATCTGTGA TAGTGACATG GAAATAGCAA ATGACAGAAG    13450
ACAAGCATTT CTCTCAAGAC ATCTTGCCTT TGTGTGTTGT TTAGCAGAGA    13500
TAGCATCTTT TGGACCAAAT TTATTAAATC TAACATATCT AGAGAGACTT    13550
GATGAATTAA AACAATACTT AGATCTGAAC ATCAAAGAAG ATCCTACTCT    13600
TAAATATGTG CAAGTATCAG GACTGTTAAT TAAATCATTC CCCTCAACTG    13650
TTACGTATGT AAGGAAAACT GCGATTAAGT ATCTGAGGAT TCGTGGTATT    13700
AATCCGCCTG AAACGATTGA AGATTGGGAT CCCATAGAAG ATGAGAATAT    13750
CTTAGACAAT ATTGTTAAAA CTGTAAATGA CAATTGCAGT GATAATCAAA    13800
AGAGAAATAA AAGTAGTTAT TTCTGGGGAT TAGCTCTAAA GAATTATCAA    13850
GTCGTGAAAA TAAGATCCAT AACGAGTGAT TCTGAAGTTA ATGAAGCTTC    13900
GAATGTTACT ACACATGGAA TGACACTTCC TCAGGAGGA AGTTATCTAT    13950
CACATCAGCT GAGGTTATTT GGAGTAAACA GTACAAGTTG TCTTAAAGCT    14000
CTTGAATTAT CACAAATCTT AATGAGGGAA GTTAAAAAAG ATAAAGATAG    14050
ACTCTTTTTA GGAGAAGGAG CAGGAGCTAT GTTAGCATGT TATGATGCTA    14100
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FIG. 6F

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CACTCGGTCC TGCAATAAAT TATTATAATT CTGGTTTAAA TATTACAGAT  14150
GTAATTGGTC AACGGGAATT AAAAATCTTC CCATCAGAAG TATCATTAGT  14200
AGGTAAAAAA CTAGGAAATG TAACACAGAT TCTTAATCGG GTGAGGGTGT  14250
TATTTAATGG GAATCCCAAT TCAACATGGA TAGGAAATAT GGAATGTGAG  14300
AGTTTAATAT GGAGTGAATT AAATGATAAG TCAATTGGTT TAGTACATTG  14350
TGACATGGAG GGAGCGATAG GCAAATCAGA AGAAACTGTT CTACATGAAC  14400
ATTATAGTAT TATTAGGATT ACATATTTAA TCGGGGATGA TGATGTTGTC  14450
CTAGTATCAA AAATTATACC AACTATTACT CCGAATTGGT CTAAAATACT  14500
CTATCTATAC AAGTTGTATT GGAAGGATGT AAGTGTAGTG TCCCTTAAAA  14550
CATCCAATCC TGCCTCAACA GAGCTTTATT TAATTTCAAA AGATGCTTAC  14600
TGTACTGTAA TGGAACCCAG TAATCTTGTT TTATCAAAAC TTAAAAGGAT  14650
ATCATCAATA GAAGAAAATA ATCTATTAAA GTGGATAATC TTATCAAAAA  14700
GGAAGAATAA CGAGTGGTTA CAGCATGAAA TCAAAGAAGG AGAAAGGGAT  14750
TATGGGATAA TGAGGCCATA TCATACAGCA CTGCAAATTT TTGGATTCCA  14800
AATTAACTTA AATCACTTAG CTAGAGAATT TTTATCAACT CCTGATTTAA  14850
CCAACATTAA TAATATAATT CAAAGTTTTA CAAGAACAAT TAAAGATGTT  14900
ATGTTCGAAT GGGTCAATAT CACTCATGAC AATAAAGAC ATAAATTAGG   14950
AGGAAGATAT AATCTATTCC CGCTTAAAAA TAAGGGGAAA TTAAGATTAT  15000
TATCACGAAG ATTAGTACTA AGCTGGATAT CATTATCCTT ATCAACCAGA  15050
TTACTGACGG GCCGTTTTCC AGATGAAAAA TTTGAAAATA GGGCACAGAC  15100
CGGATATGTA TCATTGGCTG ATATTGATTT AGAATCCTTA AAGTTATTAT  15150
CAAGAAATAT TGTCAAAAAT TACAAGAAC ACATAGGATT AATATCATAC   15200
TGGTTTTTGA CCAAAGAGGT CAAAATACTA ATGAAGCTTA TAGGAGGAGT  15250
CAAACTACTA GGAATTCCTA AACAGTACAA AGAGTTAGAG GATCGATCAT  15300
CTCAGGGTTA TGAATATGAT AATGAATTTG ATATTGATTA ATACATAAAA  15350
ACATAAAATA AAACACCTAT TCCTCACCCA TTCACTTCCA ACAAAATGAA  15400
AAGTAAGAAA AACATGTAAT ATATATATAC CAAACAGAGT TTTTCTCTTG  15450
TTTGGT                                                 15456
```

FIG. 6G

```
ACCAAACAAG AGAAGAGACT TGCTTGGGAA TATTAATTCA AATAAAAATT    50
AACTTAGGAT TAAAGAACTT TACCGAAAGG TAAGGGGAAA GAAATCCTAA    100
GACTGTAATC ATGTTGAGTC TATTCGACAC ATTCAGTGCG CGTAGGCAGG    150
AGAACATAAC AAAATCAGCT GGTGGGGCTG TTATTCCCGG GCAAAAAAAC    200
ACTGTGTCTA TATTTGCTCT TGGACCATCA ATAACAGATG ACAATGACAA    250
AATGACATTG GCTCTTCTCT TTTTGTCTCA TTCTTTAGAC AATGAAAGC     300
AGCATGCGCA AAGAGCTGGA TTTTTAGTTT CTCTGTTATC AATGGCTTAT    350
GCCAACCCAG AATTATATTT AACATCAAAT GGTAGTAATG CAGATGTTAA    400
ATATGTCATC TACATGATAG AGAAAGACCC AGGAAGACAG AAATATGGTG    450
GGTTTGTCGT CAAGACTAGA GAGATGGTTT ATGAAAGAC AACTGACTGG     500
ATGTTTGGGA GTGATCTTGA GTATGATCAA GACAATATGT TGCAAAATGG    550
TAGAAGCACT TCTACAATCG AGGATCTTGT TCATACTTTT GGATATCCAT    600
CGTGTCTTGG AGCCCTTATA ATCCAGGTTT GGATAATACT TGTTAAGGCT    650
ATAACCAGTA TATCAGGATT GAGGAAAGGA TTCTTTACTC GGTTAGAAGC    700
ATTTCGACAA GATGGAACAG TTAAATCCAG TCTAGTGTTG AGCGGTGATG    750
CAGTAGAACA AATTGGATCA ATTATGAGGT CCCAACAGAG CTTGGTAACA    800
CTCATGGTTG AAACACTGAT AACAATGAAC ACAGGCAGGA ATGACCTGAC    850
AACAATAGAA AAGAATATAC AGATTGTAGG AAACTACATC AGAGATGCAG    900
GTCTTGCTTC ATTTTTCAAC ACAATCAGAT ATGGCATTGA GACTAGAATG    950
GCAGCTCTAA CTCTGTCTAC CCTTAGACCG GACATCAACA GACTCAAGGC    1000
ACTGATAGAG CTATATCTAT CAAAGGGGCC ACGTGCTCCT TTTATATGCA    1050
TTTTGAGAGA TCCTGTGCAT GGTGAGTTTG CACCAGGCAA CTATCCTGCC    1100
CTCTGGAGTT ATGCGATGGG TGTAGCAGTT GTACAAAACA AGGCCATGCA    1150
ACAGTATGTA ACAGGAAGGT CCTATCTGGA TATTGAAATG TTCCAACTGG    1200
GTCAAGCAGT GGCACGTGAC GCCGAGTCGC AGATGAGTTC AATATTAGAG    1250
GATGAACTGG GGGTCACACA AGAAGCCAAG CAAAGCTTGA AGAAACACAT    1300
GAAGAACATC AGCAGTTCAG ATACAACCTT CTATAAGCCT ACAGGGGGAT    1350
CAGCCATAGA AATGGCAATA GATGAGGAAG CAGAGCAGCC CGAATCCAGA    1400
GGAGACCAAG ACCAAGGAGA TGAACCTCGG TCATCCATAG TTCCTTATGC    1450
ATGGGCAGAC GAAACCGGGA ATGACAACCA AACTGAATCA ACCACAGAAA    1500
TTGACAGCAT CAAAACTGAA CAAAGAAACA TCAGAGACAG GCTGAACAAA    1550
AGACTCAACG AGAAAAGGAA ACAGAGTAAC CCGGGATCAA CTGACATCAC    1600
AAACAACACA AATCAAACTG AAATAGATGA TTTATTCAGT GCATTCGGAA    1650
GCAACTAGTC ACAAAGAGAT GACCACCATC ATCAGCAACA AGTAAGAAAA    1700
ACTTAGGATT AATGGAAATT ATCCAATCCG GAGACGGAAG GACAAATCCA    1750
GAATCCAACC ACAACTCAAT CAACCAAAGA TTCATGGAAG ACAATGTTCA    1800
AAACAATCAA ATCATGGATT CTTGGGAAGA GGGATCAGGA GATAAATCAT    1850
CTGACATCTC ATCGGCCCTC GACATCATTG AATTCATACT CAACACCGAC    1900
TCCCAAGAGA ACACGGCAGA CAGCAATGAA ATCAACACAG GAGCCACAAG    1950
ACTTAGCACG ACAATCTACC AACTTGAGTC CAAAACAACA GAAACAAGCA    2000
AGGAAAATAG TGGACCAGCT AACAAAAATC GACAGTTTGG GGCATCACAC    2050
GAACGTGCCA CAGAGACAAA AGATAGAAAT GTTAATCAGA AGACTGTACA    2100
GGGAGGATAT AGGAGAGGAA GCAGCCCAGA TAGTAGAACT GAGACTATGG    2150
TCACTCGAGG AATCTCCAGA AGCAGCCCAG ATCCTAACAA TGGAACCCAA    2200
ATCCAGGAAG ATATTGATTA CAATGAAGTT GGAGAGATGG ATAAGGACTC    2250
TACTAAGAGG GAAATGCGAC AATTTAAAGA TGTTCCAGTC AAGGTATCAG    2300
GAAGTGATGC CATTCCTCCA ACAAAACAAG ATGGAGACGG TGATGATGGA    2350
```

FIG. 7A

```
AGAGGCCTGG AATCTATCAG TACATCTGAT TCAGGATATA CCAGTATAGT    2400
GACTGCCGCA ACACTAGATG ACGAAGAAGA ACTCCTTATG AAGAACAACA    2450
GGCCAAGAAA GTATCAATCA ACACCCCAGA ACAGTGACAA GGGAATTAAA    2500
AAAGGGAGTG GAAGGCCAAA AGACACAGAC AAACAATCAC CAATATTGGA    2550
CTACGAACTC AACTCCAAAG GATCGAAGAA GAGCCAGAAA ATCCTCAAAG    2600
CCAGCACGAA TACAGGAGAA CCAACAAGAT CACAGAGTGG ATCCCAGGGG    2650
AAGAGAATCA CATCCTGGAA CATCCTCAAC AGCGAGAGCG GCAATCGAGC    2700
AGAATCAACA AACCAAACCC ATCAGACATC AATCTCGGGA CAGAACCACA    2750
CAATGGGACC AAGCAGAACA ACCTCAGAAC CAAGGACCAA GACACAAAAG    2800
ACGGATGGAA AGGAAAGAGA GGACACAGAA GAGAGCACTC GATTTACAGA    2850
AAGGGCGATT ACATTATTAC AGAATCTTGG TGTAATCCAA TCTGCAGCAA    2900
AATTAGACCT ATACCAAGAC AAGAGAGTTG TGTGTGTGGC GAATGTCCTA    2950
AACAATGCAG ATACTGCATC AAAGATAGAC TTCCTAGCAG GTTTGATGAT    3000
AGGAGTGTCA ATGGATCATG ATGTCAAATT AAATCAGATT CAGAACGAGA    3050
TATTAAGTTT AAAAACTGAT CTTAAGAAGA TGGATGAATC ACATAGAAGA    3100
CTAATTGAGA ATCAAAAAGA ACAATTATCA CTGATCACAT CATTAATCTC    3150
AAATCTTAAA ATCATGACAG AGAGGAGG GAAGAAGGAC CAACCAGAAC    3200
CTAGCGGGAG GACATCCATG ATCAAGACAA AGGCAAAAGA AGAGAGAATA    3250
AAGAAAGTCA GGTTTGACCC TCTTATGGAA ACACAGGGCA TCGAGAAAAA    3300
CATCCCTGAC CTCTACAGAT CAATAGAGAA AACACCAGAA AACGACACAC    3350
AGATCAAATC AGAAATAAAC AGATTGAATG ATGAATCCAA TGCCACTAGA    3400
TTAGTACCTA GAAGAATAAG CAGTACAATG AGATCACTAA TAATAATCAT    3450
CAACAACAGC AATTTATCAT CAAAAGCAAA GCAATCATAC ATCAACGAAC    3500
TCAAGCTCTG CAAGAGTGAT GAGGAAGTGT CTGAGTTGAT GGACATGTTC    3550
AATGAGGATG TCAGCTCCCA GTAAACCGCC AACCAAGGGT CAACACCAAG    3600
AAAACCAACA GCACAAAACA GCCAATAAGA GACCATCCCA ACACACCGAA    3650
CCAATCAACA CATAACAAAG ATCTTTAGAT CATAGATGAC TAAGAAAAAC    3700
TTAGGATGAA AGGACTGATC AATCCTCCAA AACAATGAGC ATCACCAGCT    3750
CCACAATCTA CACATTCCCA GAATCCTCTT TCTCCGAGAA TGGCAACATA    3800
GAGCCGTTAC CACTCAAGGT CAATGAACAG AGAAAGGCCA TACCTCATAT    3850
TAGGGTTGTC AAGATAGGAG ATCCGCCCAA ACATGGATCC AGATATCTGG    3900
ATGTCTTTTT ACTGGGCTTC TTTGAAATGG AAAGGTCAAA AGACAGGTAT    3950
GGGAGCATAA GTGATCTAGA TGATGATCCA AGTTACAAGG TTTGTGGCTC    4000
TGGATCATTG CCACTTGGGT TGGCTAGATA CACTGGAAAT GATCAGGAAC    4050
TCCTACAGGC TGCAACCAAG CTCGATATAG AAGTAAGAAG AACTGTAAAG    4100
GCTACGGAGA TGATAGTTTA CACTGTGCAA AACATCAAAC CTGAACTATA    4150
TCCATGGTCC AGTAGATTAA GAAAAGGGAT GTTATTTGAC GCTAACAAGG    4200
TTGCACTTGC TCCTCAATGT CTTCCACTAG ATAGAGGGAT AAAATTCAGG    4250
GTGATATTTG TGAACTGCAC AGCAATTGGA TCAATAACTC TATTCAAAAT    4300
CCCCAAGTCC ATGGCATTGT TATCATTGCC TAATACAATA TCAATAAATC    4350
TACAAGTACA TATCAAAACA GGAATTCAGA CAGATTCCAA AGGAGTAGTT    4400
CAGATTCTAG ATGAAAAGG TGAAAAATCA CTAAATTTCA TGGTTCATCT    4450
CGGGTTGATC AAAAGGAAGA TGGGTAGAAT GTACTCAGTT GAATATTGTA    4500
AGCAGAAGAT TGAGAAGATG AGATTATTAT TCTCATTGGG ATTAGTTGGA    4550
GGGATCAGCT TCCACGTCAA CGCAACTGGC TCTATATCAA AGACATTAGC    4600
AAGTCAATTA GCATTTAAAA GAGAAATCTG CTATCCCCTA ATGGATCTGA    4650
ATCCACACTT AAATTTAGTT ATATGGGCAT CATCAGTTGA AATTACAAGA    4700
```

FIG. 7B

```
GTAGATGCAA  TTCTCCAGCC  TTCATTACCT  GGCGAATTCA  GATACTACCC     4750
AAACATCATA  GCAAAAGGGG  TCGGGAAAAT  CAGACAGTAA  AACCAACAAC     4800
CCTGACATCC  AACACTGCAA  ATCAGGCTAC  CCACAGGAGA  AAAATCAAAA     4850
ACTTAGGATC  AAAGGGATCA  CCACAAACCC  CGGGAAACAG  CCAAACCAAC     4900
CAACACACAA  ATCACAGACA  AAAAGGAAAA  GGCACTGCAA  AGACCGAGAA     4950
CAAGCAGAAC  GCACACAACC  AAGCAGAGGA  AAGCCAAAGC  CCGCCATTCA     5000
CAAACACACC  AACAATCCTA  CAAACAAGCA  CCAAAATAGA  GGTCAAAAGA     5050
CAAAGAGCAT  CAGATATGAC  CATCACAACC  ATAATCATAG  CCATACTACT     5100
AATACCCCTA  TCATTCTGTC  AAATAGACAT  AACAAAACTG  CAACGTGTAG     5150
GTGTATTAGT  CAACAATCCC  AAAGGCATGA  AAATTTCACA  AAATTTTGAA     5200
ACGAGATACC  TGATATTAAG  TCTGATACCC  AAAATAGAGA  ATTCACACTC     5250
ATGTGGGGAT  CAACAGATAA  ACCAATACAA  GAAGTTATTG  GATAGATTGA     5300
TAATTCCTCT  ATATGATGGA  TTAAAATTAC  AAAAGATGT   AATAGTAGTA     5350
AGTCATGAAA  CCCATAATAA  TACTAATCTT  AGGACAAAAC  GATTCTTTGG     5400
AGAGATAATT  GGGACAATTG  CGATAGGGAT  AGCCACCTCA  GCGCAAATCA     5450
CCGCAGCAGT  CGCTCTTGTC  GAAGCTAAAC  AGGCAAGGTC  AGACATAGAA     5500
AAACTCAAAG  AAGCTATAAG  AGACACAAAC  AAGGCAGTAC  AATCGATTCA     5550
AAGTTCTGTA  GGTAACCTAA  TTGTTGCAGT  TAAATCAGTT  CAAGACTATG     5600
TCAACAATGA  AATTGTACCT  TCAATCACAA  GATTAGGCTG  TGAAGCAGCA     5650
GGGTTACAAT  TGGGAATTGC  ACTGACACAA  CATTACTCAG  AATTAACAAA     5700
TATATTTGGT  GATAATATAG  GAACACTGAA  AGAAAAGGG   ATAAAATTAC     5750
AGGGGATAGC  ATCGTTATAT  CATACAAACA  TAACAGAAAT  ATTTACTACT     5800
TCAACAGTTG  ACCAATATGA  TATTTATGAC  CTATTATTCA  CTGAATCAAT     5850
CAAGATGAGA  GTGATAGATG  TTGATTTGAG  TGATTACTCA  ATTACTCTTC     5900
AAGTTAGACT  TCCTTTATTA  ACTAAACTAT  CAAATACTCA  GATTTATAAA     5950
GTAGATTCTA  TATCATACAA  CATCCAGGGC  AAAGAGTGGT  ATATTCCTCT     6000
TCCCAATCAC  ATCATGACAA  AAGGGGCTTT  TCTAGGTGGT  GCTGATATTA     6050
AAGAATGCAT  AGAGGCATTC  AGCAGTTATA  TATGTCCTTC  TGATCCAGGT     6100
TATATATTAA  ATCACGAGAT  AGAGAATTGT  TTATCAGGA   ACATAACACA     6150
GTGTCCTAAG  ACTGTTGTTA  CATCAGATGT  GGTACCACGA  TACGCGTTTG     6200
TGAATGGTGG  ATTAATTGCA  AACTGCATAA  CAACTACATG  TACATGCAAT     6250
GGAATTGACA  ATAGAATTAA  TCAATCACCT  GATCAAGGAA  TTAAGATCAT     6300
AACACATAAA  GAATGCCAGG  TAATAGGTAT  AAACGGAATG  TTATTCAATA     6350
CTAATAGAGA  AGGGACATTA  GCAACTTATA  CATTTGATGA  CATTATATTA     6400
AATAACTCTG  TTGCACTTAA  TCCAATTGAT  ATATCTATGG  AACTTAACAA     6450
GGCAAAACTA  GAATTAGAAG  AATCGAAGGA  ATGGATAAAG  AAATCAAATC     6500
AAAAGTTAGA  TTCCGTTGGA  AGTTGGTATC  AATCTAGTGC  AACAATCACC     6550
ATAATCATAG  TGATGATAAT  AATTCTATTT  ATAATCAATA  TAACAATTAT     6600
TGTAGTCATA  ATCAAATTCT  ATAGAATTAA  GGGGGAAAAT  CAAAACGACA     6650
AAAACAGTGA  GCCGTATATA  CTGACAAATA  GACAATAAGA  CTATACACGA     6700
TCAAATATAG  AAAGTACAAA  AAACTTAGGA  ACAAAGTTGT  TCAACACAGC     6750
AGCAGCGAAC  AGACCCAAAG  GCAGCGCAGA  GGCGACACCG  AACCCAAAAA     6800
TGGAATATTG  GAAACACACA  AACAGCACAA  AAAACACCAA  CAATGAAACC     6850
GAAACAACCA  GAGGCAAACA  CAGTAGCAAG  GTTACAAATA  TCATAATGTA     6900
CACCTTCTGG  ACAATAACAT  CAACAATATT  ATTAGTCATT  TTTATAATGA     6950
TATTGACAAA  CTTAATTCAA  GAGAACAATC  ATAATAAATT  AATGTTGCAG     7000
GAAATAAGAA  AAGAATTCGC  GGCAATAGAC  ACCAAGATTC  AGAGGACCTC     7050
```

## FIG. 7C

```
GGATGACATT  GGAACCTCAA  TACAGTCAGG  AATAAATACA  AGACTTCTCA   7100
CAATTCAGAG  TCATGTTCAA  AACTATATCC  CACTATCACT  AACACAACAA   7150
ATGTCAGATC  TCAGAAAATT  TATCAATGAT  CTAACAAATA  AAAGAGAACA   7200
TCAAGAAGTG  CCAATACAGA  GAATGACTCA  TGATAGAGGT  ATAGAACCCC   7250
TAAATCCAGA  CAAGTTCTGG  AGGTGTACAT  CTGGTAACCC  ATCTCTAACA   7300
AGTAGTCCTA  AGATAAGGTT  AATACCAGGG  CCAGGTTTAT  TAGCAACATC   7350
TACTACAGTA  AATGGCTGTA  TTAGAATCCC  ATCGTTAGCA  ATCAATCATT   7400
TAATCTACGC  TTACACCTCT  AATCTTATCA  CCCAGGGCTG  TCAAAATATA   7450
GGGAAATCTT  ACCAAGTACT  ACAAATAGGG  ATAATTACTA  TAAATTCGGA   7500
CCTAGTACCT  GATTTAAATC  CCAGAGTCAC  ACATACATTT  AATATTGATG   7550
ATAATAGGAA  ATCTTGCTCT  CTGGCACTAT  TGAATACAGA  TGTTTATCAG   7600
TTATGCTCAA  CACCAAAGT  TGATGAGAGA  TCCGATTATG  CATCAACAGG   7650
TATTGAGGAT  ATTGTACTTG  ACATTGTCAC  TAATAATGGA  TTAATTATAA   7700
CAACAAGGTT  TACAAATAAT  AATATAACTT  TTGATAAACC  GTATGCAGCA   7750
TTGTATCCAT  CAGTAGGACC  AGGAATCTAT  TATAAGGGTA  AAGTTATATT   7800
TCTCGGATAT  GGAGGTCTAG  AGCATGAAGA  AAACGGAGAC  GTAATATGTA   7850
ATACAACTGG  TTGTCCTGGC  AAAACACAGA  GAGACTGTAA  TCAGGCTTCT   7900
TATAGCCCAT  GGTTCTCAAA  TAGGAGAATG  GTAAACTCTA  TTATTGTTGT   7950
TGATAAAGGC  ATAGATGCAA  CTTTTAGCTT  GAGGGTGTGG  ACTATTCCAA   8000
TGAGCCAAAA  TTATTGGGGA  TCAGAAGGAA  GATTACTTTT  ATTAGGTGAC   8050
AGAATATACA  TATATACTAG  ATCCACAAGT  TGGCACAGTA  AATTACAGTT   8100
AGGGGTAATT  GATATTCTG  ATTATAATAA  TATAAGAATA  AATTGGACTT   8150
GGCATAATGT  ACTATCACGG  CCAGGAAATG  ATGAATGTCC  ATGGGGTCAT   8200
TCATGCCCAG  ACGGATGTAT  AACAGGAGTT  TACACTGATG  CATATCCGCT   8250
AAACCCATCG  GGGAGTGTTG  TATCATCAGT  AATTCTTGAC  TCACAAAAGT   8300
CTAGAGAAAA  CCCAATCATT  ACCTACTCAA  CAGCTACAAA  TAGAATAAAT   8350
GAATTAGCTA  TATATAACAG  AACACTTCCA  GCTGCATATA  CAACAACAAA   8400
TTGTATCACA  CATTATGATA  AAGGGTATTG  TTTTCATATA  GTAGAAATAA   8450
ATCACAGAAG  TTTGAATACG  TTTCAACCTA  TGTTATTCAA  AACAGAAGTT   8500
CCAAAAAACT  GCAGCTAAAT  TGATCATCGC  ATATCGGATG  CCAGATGACA   8550
TTAAAAGAGA  CCACCAGACA  GACAACACAG  GAGATGATGC  AAGATATAAA   8600
GGAATAATAA  AAAACTTAGG  AGAAAAGTGT  GCAAGAAAAA  TGGACACTGA   8650
ATCCCACAGC  GGCACAACAT  CTGACATTCT  GTACCTGAA   TGTCACCTCA   8700
ATTCTCCTAT  AGTTAAAGGA  AAAATAGCAC  AACTGCATAC  AATAATGAGT   8750
TTGCCCCAAC  CCTACGATAT  GGATGATGAT  TCAATACTGA  TTATTACTAG   8800
ACAAAAAATC  AAACTCAATA  AATTAGATAA  AAGACAACGG  TCAATTAGGA   8850
AATTAAGATC  AGTCTTAATG  GAAAGAGTAA  ATGATCTTGG  TAAATACACC   8900
TTTATCAGAT  ATCCAGAAAT  GTCTAGTGAA  ATGTTCCAAT  TATGTATACC   8950
CGGAATTAAT  AATAAAATAA  ATGAATTGCT  AAGTAAAGCA  AGTAAAACAT   9000
ATAATCAAAT  GACTGATGGA  TTAAGAGATC  TATGGGTTAC  TGTACTATCG   9050
AAGTTAGCAT  CGAAAAATGA  TGGAAGTAAT  TATGATATCA  ATGAAGATAT   9100
TAGCAATATA  TCAAATGTTC  ACATGACTTA  CCAATCAGAC  AAATGGTATA   9150
ATCCATTCAA  GACATGGTTT  ACTATTAAGT  ATGACATGAG  GAGATTACAA   9200
AAAGCCAAAA  ATGAGATTAC  ATTCAATAGG  CATAAAGATT  ATAATCTATT   9250
AGAAGACCAA  AAGAATATAT  TGCTGATACA  TCCAGAACTC  GTCTTAATAT   9300
TAGATAAACA  AAATTACAAT  GGGTATATAA  TGACTCCTGA  ATTGGTACTA   9350
ATGTATTGTG  ATGTAGTTGA  AGGGAGGTGG  AATATAAGTT  CATGTGCAAA   9400
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FIG. 7D

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ATTGGATCCT AAATTACAAT CAATGTATTA TAAAGGTAAC AATTTATGGG    9450
AAATAATAGA TGGACTATTC CTGACCTTAG GAGAAAGAAC ATTTGACATA    9500
ATATCACTAT TAGAACCGCT TGCATTATCG CTCATTCAAA CTCATGACCC    9550
GGTTAAACAG CTCAGAGGGG CTTTTTTAAA TCACGTGTTA TCAGAAATGG    9600
AATCAATATT CGCAGCTGAG TGTACAACAG AGGAAATACC TAATGTGGAT    9650
TATATAGATA AAATTTTAGA TGTATTCAAA GAATCAACAA TAGATGAAAT    9700
AGCAGAAATT TTCTCTTTCT CCGAACTTT TGGACACCCT CCATTAGAGG    9750
CGAGTATAGC AGCAGAGAAA GTTAGAAAGT ATATGTACAC TGAGAAATGT    9800
TTGAAATTTG ATACTATCAA TAAATGTCAT GCTATTTTTT GTACAATAAT    9850
TATAAATGGA TATAGAGAAA GACATGGTGG TCAATGGCCT CCAGTTACAT    9900
TACCTATTCA TGCACATGAA TTTATCATAA ATGCGTACGG ATCAAATTCT    9950
GCCATATCAT ATGAAAATGC TGTAGATTAT TATAAGAGCT TCATAGGAAT   10000
AAAATTTGAC AAGTTTATAG AGCCTCAATT GGATGAAGAC TTAACTATTT   10050
ATATGAAAGA TAAAGCATTA TCCCCAAAGA AATCTAACTG GACACAGTC    10100
TATCCAGCTT CAAACCTGTT ATACCGCACT AATGTGTCTC ATGATTCACG   10150
AAGATTGGTT GAAGTATTTA TAGCAGATAG TAAATTTGAT CCCCACCAAG   10200
TATTAGATTA CGTAGAATCA GGATATTGGC TAGATGATCC TGAATTTAAT   10250
ATCTCATATA GTTTAAAAGA GAAAGAAATA AAACAAGAAG GTAGACTTTT   10300
TGCAAAAATG ACATACAAGA TGAGAGCTAC ACAAGTATTA TCAGAAACAT   10350
TATTGGCGAA TAATATAGGG AAATTCTTCC AAGAGAATGG GATGGTTAAA   10400
GGAGAAATTG AATTACTCAA GAGACTGACA ACAATATCTA TGTCTGGGGT   10450.
TCCGCGGTAT AATGAGGTAT ACAATAATTC AAAAAGTCAC ACAGAGGAAC   10500
TTCAAGCTTA TAATGCAATT AGCAGTTCCA ATTTATCTTC TAATCAGAAG   10550
TCAAAGAAGT TTGAATTTAA ATCAACAGAT ATATACAATG ATGGATACGA   10600
AACCGTAAGC TGCTTCTTAA CGACAGATCT TAAAAAATAT TGTTTAAATT   10650
GGAGGTATGA ATCAACAGCT TTATTCGGTG ATACTTGTAA TCAGATATTT   10700
GGGTTAAAGG AATTATTTAA TTGGCTGCAC CCTCGCCTTG AAAAGAGTAC   10750
AATATATGTT GGAGATCCTT ATTGCCCGCC ATCAGATATT GAACATTTAC   10800
CACTTGATGA CCATCCTGAT TCAGGATTTT ATGTTCATAA TCCTAAAGGA   10850
GGAATAGAAG GGTTTTGCCA AAAGTTATGG ACACTCATAT CTATCAGTGC   10900
CATACATTTA GCAGCTGTCA AAATCGGTGT AAGAGTTACT GCAATGGTTC   10950
AAGGGGATAA TCAAGCCATA GCTGTTACCA CCAGAGTACC TAATAATTAT   11000
GATTATAAGG TTAAGAAAGA GATTGTTTAT AAAGATGTGG TAAGATTTTT   11050
TGATTCTTTG AGAGAGGTTA TGGATGATCT GGGTCATGAG CTCAAACTAA   11100
ATGAAACTAT AATAAGTAGT AAAATGTTTA TATATAGCAA AAGGATATAC   11150
TATGACGGAA GAATCCTTCC TCAGGCGTTA AAAGCATTGT CTAGATGTGT   11200
TTTTTGGTCT GAAACAATCA TAGATGAGAC AAGATCAGCA TCCTCAAATC   11250
TGGCGACATC GTTTGCAAAG GCCATTGAGA ATGGCTACTC ACCTGTATTG   11300
GGATATGTAT GCTCAATCTT CAAAAATATC CAACAGTTGT ATATAGCACT   11350
TGGAATGAAT ATAAATCCAA CTATAACCCA AAATATTAAA GATCAATATT   11400
TCAGGAATAT TCATTGGATG CAATATGCAT CTCTAATCCC TGCTAGTGTC   11450
GGAGGATTTA ATTATATGGC CATGTCAAGG TGTTTTGTCA GAAACATTGG   11500
AGATCCTACA GTCGCTGCAT TAGCTGATAT TAAAAGATTT ATAAAAGCAA   11550
ATTTGTTAGA TCGAGGTGTC CTTTACAGAA TTATGAATCA GGAACCAGGC   11600
GAGTCCTCCT TTTTAGACTG GGCTTCAGAC CCCTATTCAT GTAACTTACC   11650.
ACAATCTCAA AATATAACCA CCATGATAAA GAATATAACT GCAAGAAATG   11700
TACTACAGGA CTCACCAAAC CCATTACTAT CTGGATTATT TACAAGTACA   11750
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FIG. 7E
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ATGATAGAAG  AGGATGAGGA  ATTAGCTGAG  TTCCTAATGG  ACAGGAGAAT  11800
AATTCTCCCA  AGGGTTGCGC  ATGACATTTT  AGATAATTCT  CTTACTGGAA  11850
TTAGGAATGC  TATAGCTGGT  ATGTTGGATA  CAACAAAATC  ACTAATTCGA  11900
GTAGGGATAA  ACAGAGGAGG  ATTAACCTAT  AACTTATTAA  GAAAGATAAG  11950
CAACTATGAT  CTTGTACAAT  ATGAGACACT  TAGTAAAACT  TTAAGACTAA  12000
TAGTCAGTGA  CAAGATTAAG  TATGAAGATA  TGTGCTCAGT  AGACCTAGCC  12050
ATATCATTAA  GACAAAAAAT  GTGGATGCAT  TTATCAGGAG  GAAGAATGAT  12100
AAATGGACTT  GAAACTCCAG  ATCCTTTAGA  GTTACTGTCT  GGAGTAATAA  12150
TAACAGGATC  TGAGCATTGT  AGGATATGTT  ATTCAACTGA  AGGTGAAAGC  12200
CCATATACAT  GGATGTATTT  ACCAGGCAAT  CTTAATATAG  GATCAGCTGA  12250
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FIG. 7F

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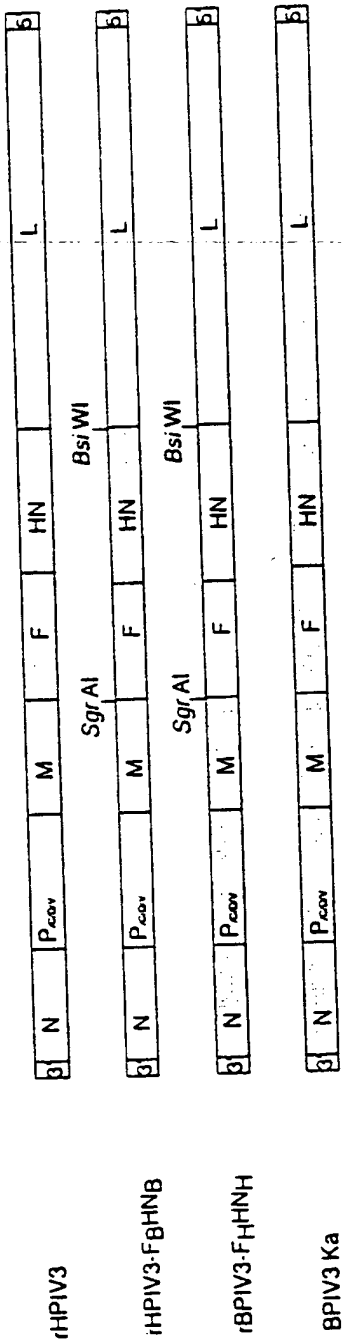```

FIG. 7G

# Figure 8A

Figure 8B

Assembly of an antigenomic cDNA for BPIV3 Ka

Figure 8C

Generation of full length cDNA clones encoding HPIV3/BPIV3 antigenic chimeric viruses

1. Generation of HPIV3 and BPIV3 full length clones

2. Mutagenesis to create unique SgrAI and BsiWI restriction sites

3. Cloning of the F and HN genes into the heterologous full length cDNA

# Figure 9

Figure 10

# Figure 11

SEQUENCE LISTING

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<150> 60/143,134
<151> 1999-07-09

<160> 31

<170> PatentIn Ver. 2.1

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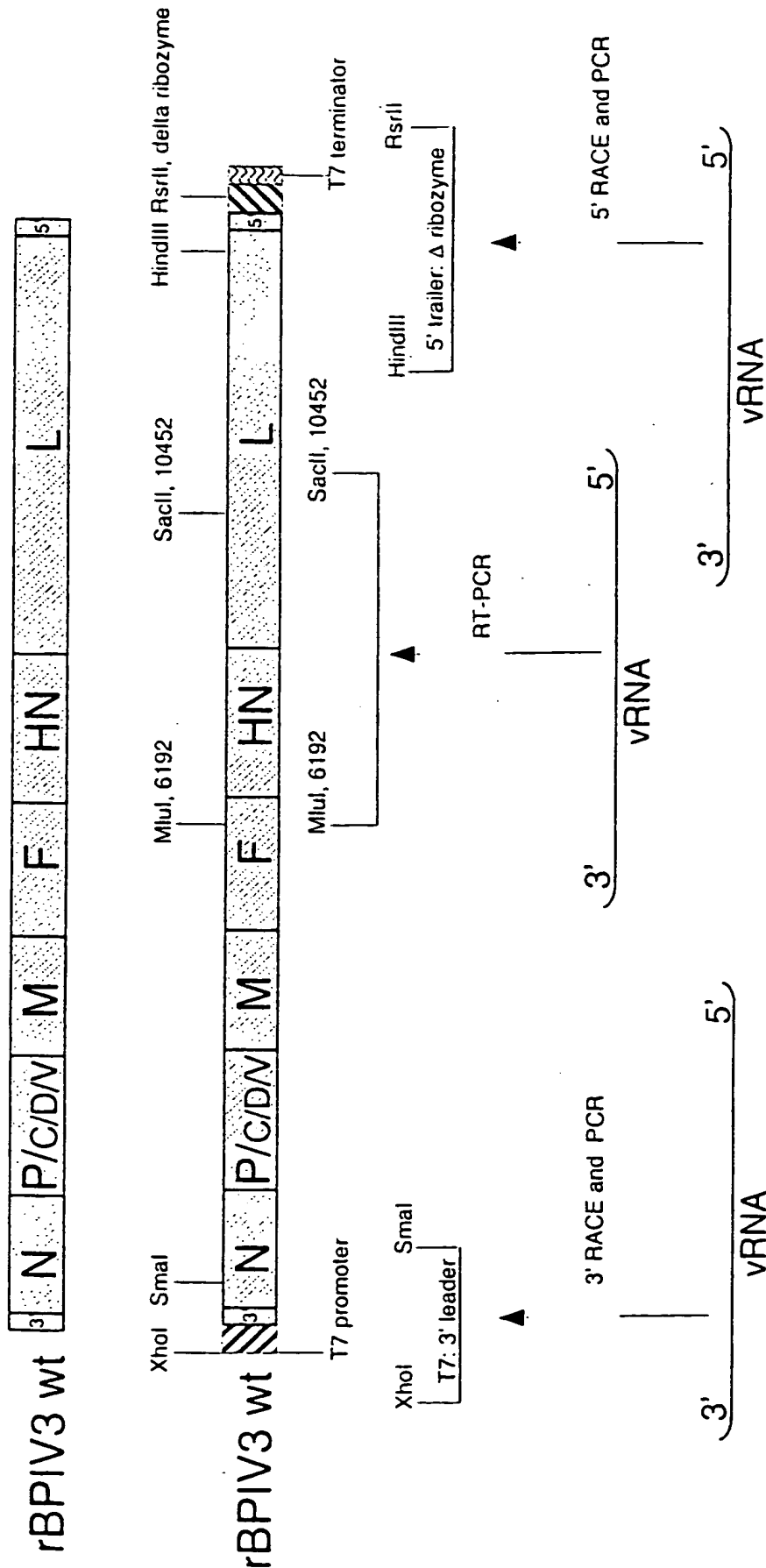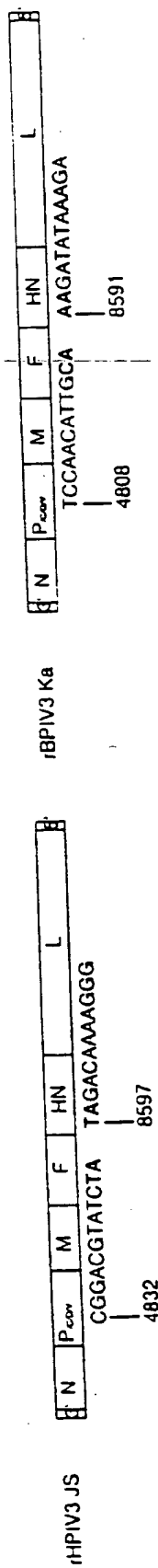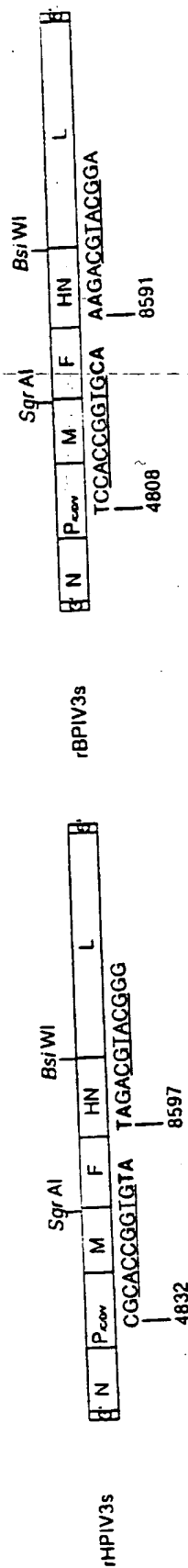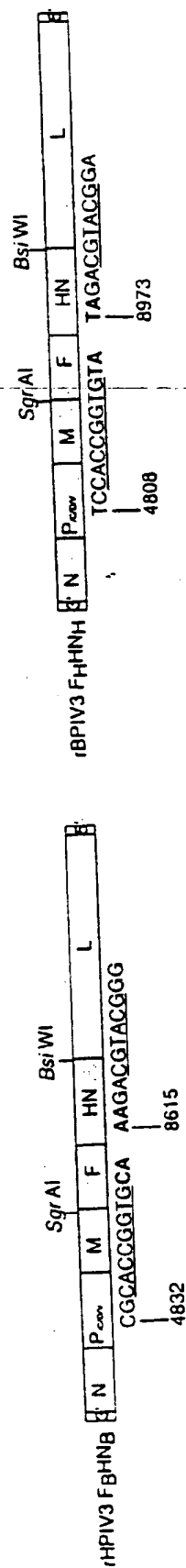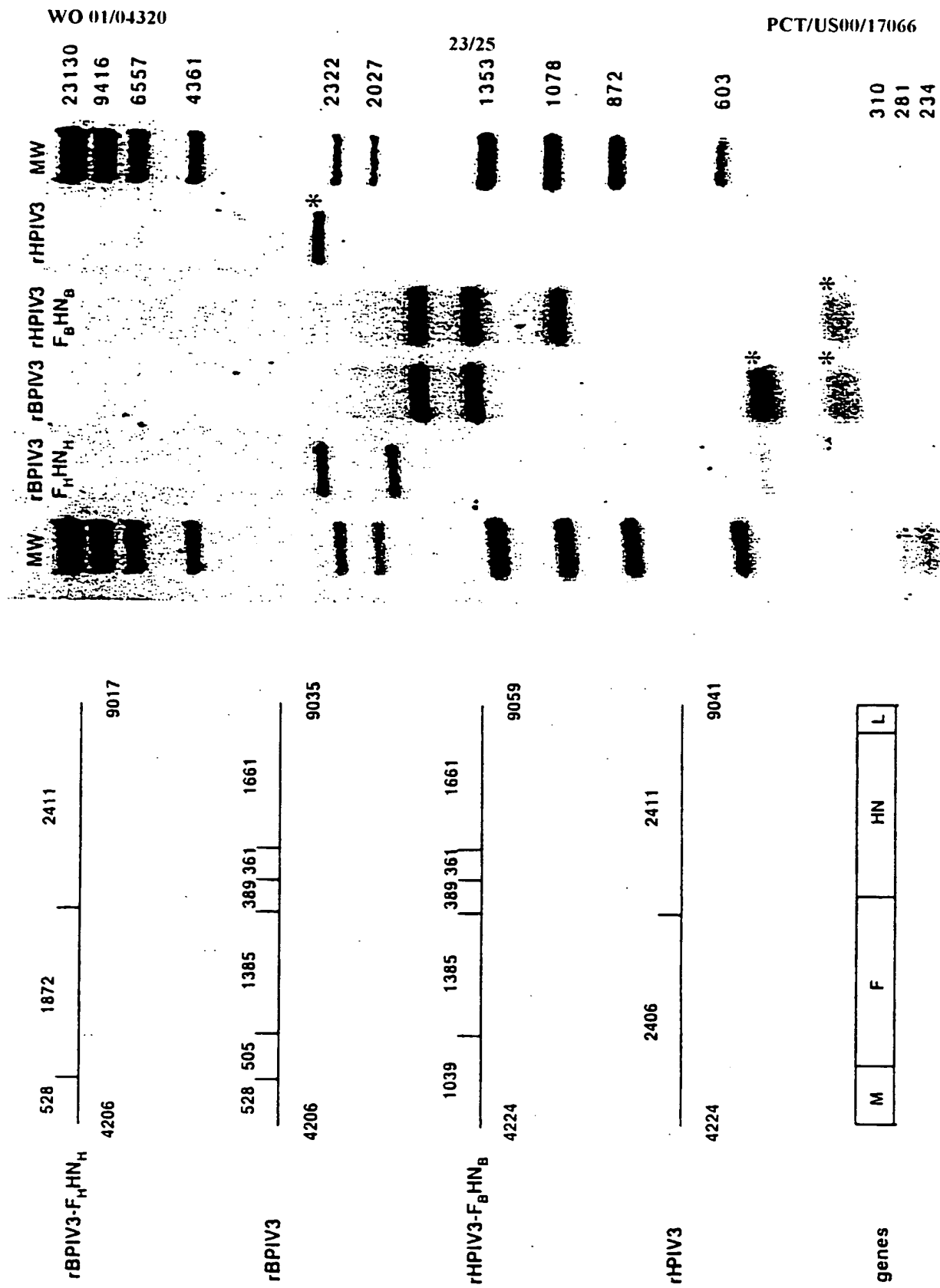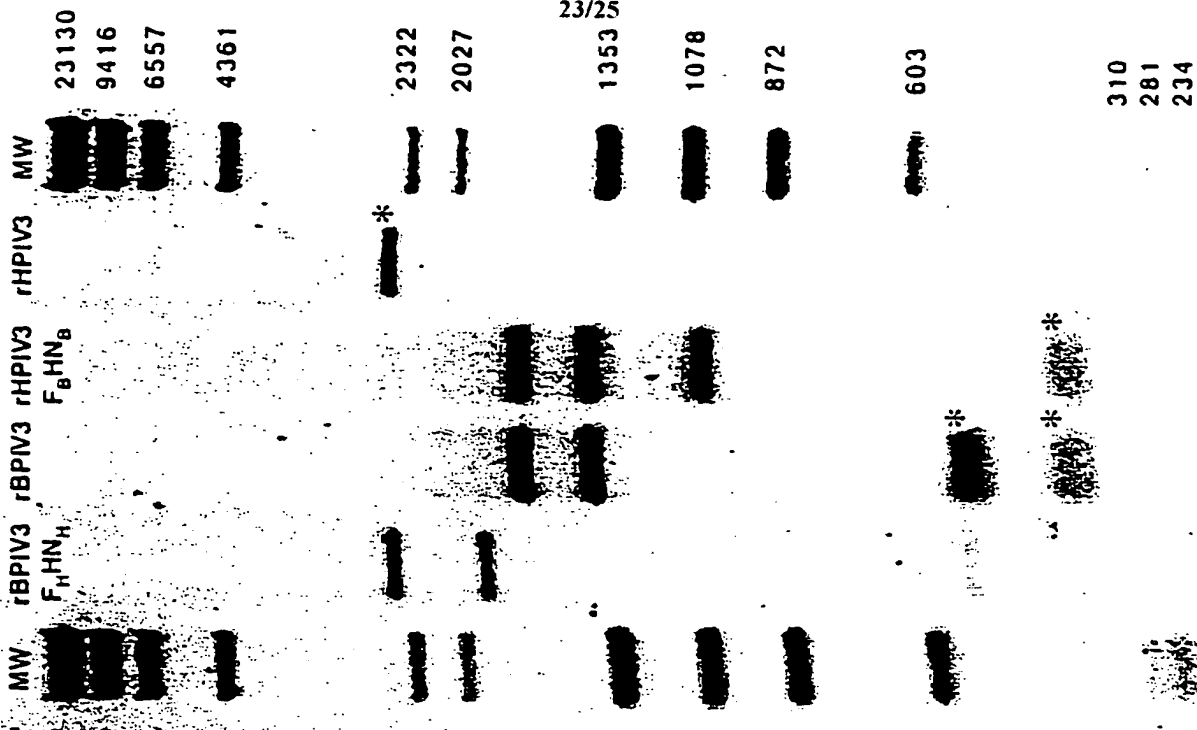<211> 12
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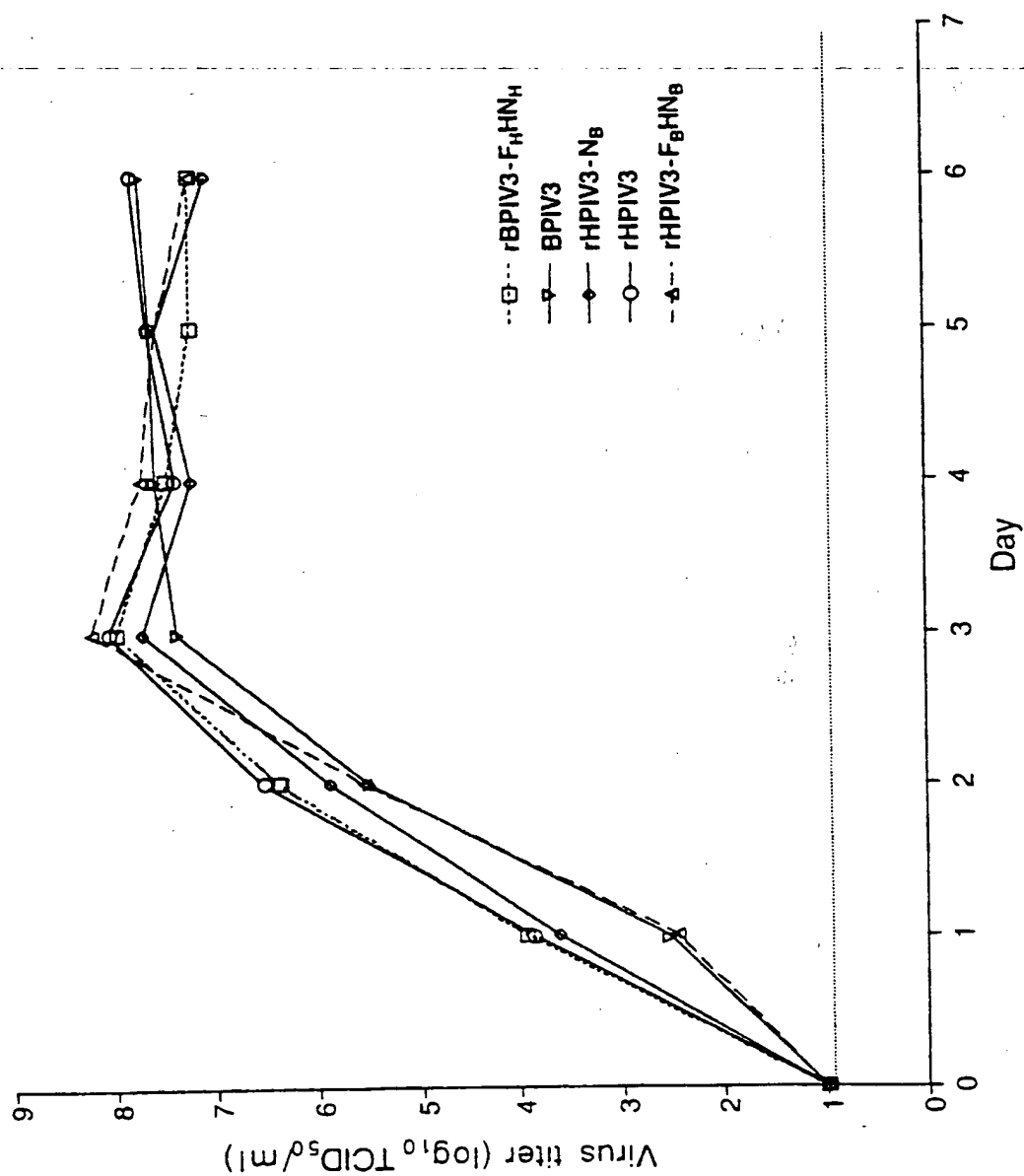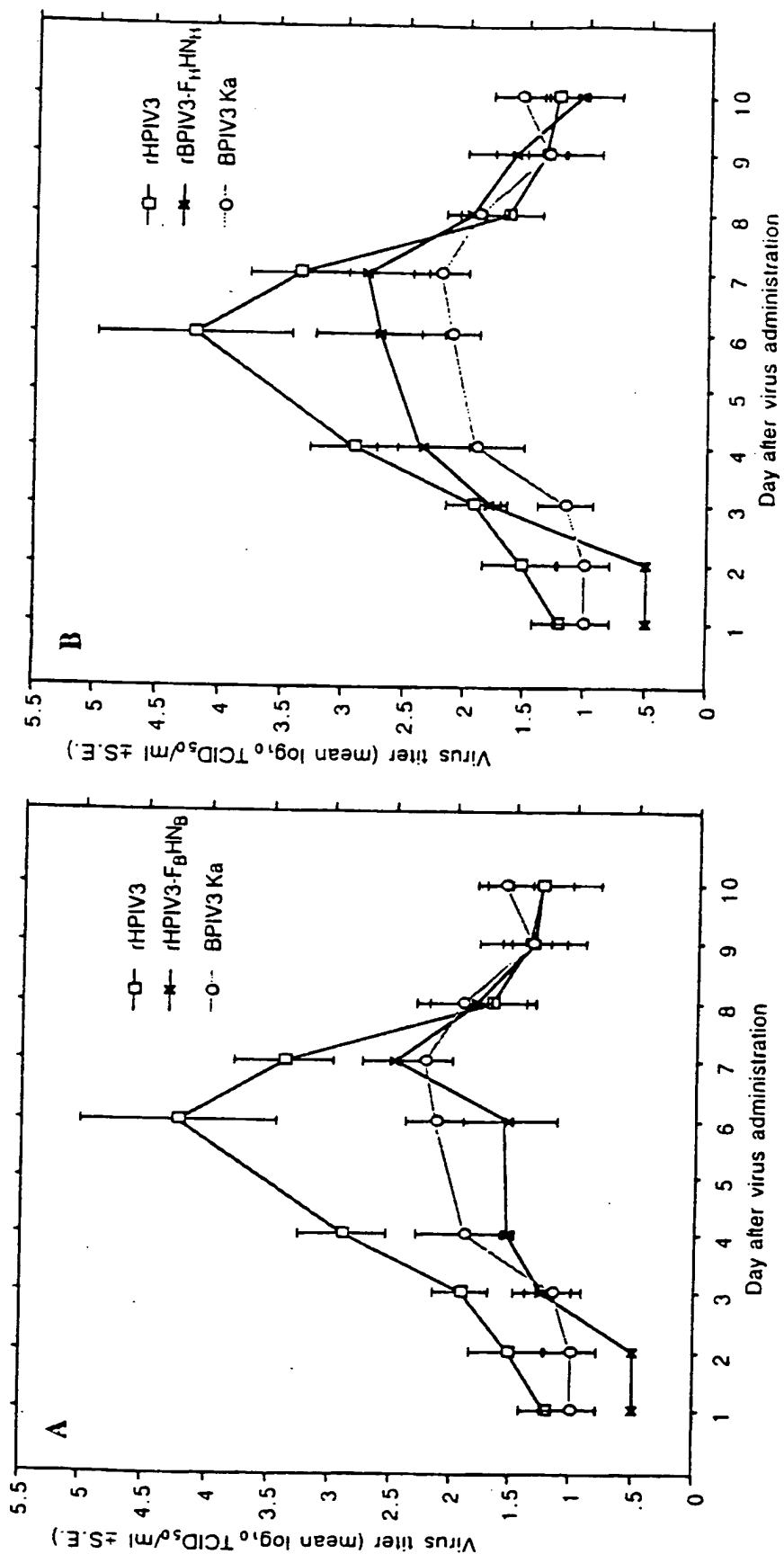
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<210> 5
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<210> 6
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flanking site for introduction of Bsi W1 site for
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3

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<210> 11
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<210> 12
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
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      start codon

<400> 12
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<210> 13
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<220>
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4

flanking introduced restriction site at N gene
stop codon

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<210> 14
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flanking mutation to restore context for N gene
start codon

<400> 14
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stop codon

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<210> 18
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<210> 19
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6

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<210> 22
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7

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13

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# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7     C12N15/45     C12N5/10     C12N7/00     C07K14/115     A61K39/155

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7     C12N     C07K     A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | BAILLY J.E. ET AL.: "A recombinant human parainfluenza virus type 3 (PIV3) in which the nucleocapsid N protein has been replaced by that of bovine PIV3 is attenuated in primates." J. VIROL., vol. 74, no. 7, April 2000 (2000-04), pages 3188-3195, XP002149310 the whole document --- | 1-83 |
| X | WO 98 53078 A (DURBIN ANNA P ;TAO TAO (US); COLLINS PETER L (US); MURPHY BRIAN R) 26 November 1998 (1998-11-26) cited in the application the whole document see in particular p. 36, lines 15-30. --- | 1-83 |

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[X] Further documents are listed in the continuation of box C.     [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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